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Klebsiella pneumoniae: a progression to multidrug resistance

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I. Abstract.

Klebsiella pneumoniae is a common cause of nosocomial and community-acquired infections, and the increasing incidence and prevalence of antibiotic resistant strains is proving to be particularly problematic to clinicians. *K. pneumoniae* is capable of employing a multitude of mechanisms by which to confer resistance to most available antibiotics. The carbapenem antibiotics are usually reserved for the treatment of complicated or multidrug resistant (MDR) *K. pneumoniae* infections. The recent emergence of not only MDR but also pan-drug resistant (PDR) *K. pneumoniae* strains has signified that it is now more important than ever to understand the mechanisms by which these strains confer resistance so that we may find ways to combat or hinder this progression. This project aimed to investigate the regulation of the transcriptional activator RamA, its ability to confer a MDR phenotype, and the mechanisms employed by *K. pneumoniae* to confer levels of carbapenem resistance sufficient to result in therapy failure.

The analysis of a panel of *K. pneumoniae* strains, containing both RamA expressers and non-expressers, demonstrated that the overexpression of RamA was sufficient to confer an MDR phenotype. Two compounds, chlorpromazine (CPZ) and tigecycline, were shown to act as inducers of *ramA*, *romA* and *acrA* transcription. CPZ exhibited synergy with the antibiotics chloramphenicol, norfloxacin and tetracycline, all of which are known substrates of the AcrAB efflux pump. The current lack of novel classes of antimicrobials in development indicate a potential for a compound, such as CPZ, to be developed and exploited for clinical use. The ability of both CPZ and

tigecycline to cause mutations within *ramR* however, indicate that both compounds may have the ability to select for efflux mutants as a result of their ability to upregulate *ramA*, which in turn causes the upregulation of the AcrAB efflux pump.

The regulation of RamA by the upstream gene *ramR,* which encodes a TetR family protein was investigated in *K. pneumoniae* isolates. Sequencing of the *ramR* genes revealed that strains exhibiting an MDR phenotype commonly contained mutations within their gene sequences. The complementation of a wildtype *ramR* into a strain containing a 32 amino acid deletion within its *ramR*, was shown to increase susceptibility to various antibiotics of different classes, and additionally downregulate the expression of *ramA*, *romA* and *acrA*. CPZ, ciprofloxacin and tigecycline *K. pneumoniae* mutants were shown to exhibit increased MICs to a broad spectrum of antibiotics with respect to their parent strains, and possess mutations within their *ramR* genes. Complementation of the wildtype *ramR* resulted in partial reversion to the parental phenotypes, indicating another mechanism must also be involved in conferring the MDR phenotypes. These studies indicated that RamR plays an important role as a negative regulator of RamA, but also that it is not the sole regulator.

The development of reduced susceptibility to the carbapenems was investigated in two clinical strains of *K. pneumoniae*, K1 and K2, isolated from the urine of a single patient at different stages of antibiotic therapy. The strains were shown to exhibit similar resistance phenotypes with the exception of their susceptibilities to the carbapenems. PCR and phenotypic analyses revealed that neither strain contained

any carbapenemases or AmpC enzymes, but both contained OXA-1, SHV-1 TEM-1 and CTX-M-15. Analysis of their OMP profiles indicated that both strains lacked OmpK35, and K2 additionally lacked OmpK36. Mutation studies showed that the phenotype and OMP profile exhibited by K2 could be achieved in K1 via single step mutations using ertapenem, imipenem or meropenem. Susceptibility testing of CTX-M-15 clinical strains showed that strains containing CTX-M-15 showed reduced activity against ertapenem in the presence of clavulanic acid. These studies indicated a potential role for CTX-M-15 in conferring reduced susceptibility to the carbapenems when found in conjunction with altered permeability and active efflux.

The mechanisms of antibiotic resistance employed by *K. pneumoniae* are numerous and complex. This work highlights several of these mechanisms and, more importantly, how they can work in synergy with one another to devastating consequences.

II. Declaration.

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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V. Publications and presentations from this thesis.

Findlay J, Schneiders T, Amyes SGB. (2008) The effect of chlorpromazine on the multidrug resistance regulator RamA. *The 48th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington DC, USA (25 – 28th October) Abstract no. 1943

Findlay J, Schneiders T. (2009) The differential effect of mutations in RamR, in mediating antibiotic susceptibility in *Klebsiella pneumoniae*. *The 19th European Congress on Clinical Microbiology and Infectious Diseases*, Helsinki, Finland (16 – 19th May) Abstract no. P1483

Findlay J, Schneiders T. (2009) RamR: A dual regulator of antibiotic susceptibility and biofilm formation in *Klebsiella pneumoniae* Kp342. *The 19th European Congress on Clinical Microbiology and Infectious Diseases*, Helsinki, Finland (16 – 19th May) Abstract no. O206

Findlay J, Hamouda A, Dancer S, Amyes SGB. (2010) Mechanisms of carbapenem resistance emerging during therapy in a strain of *Klebsiella pneumoniae* treated with meropenem. *The 50th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Boston, USA (12 – 15th September) Abstract no. 2197

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Other publications and presentations conducted during the tenure of this thesis

Evans BA, Hamouda A, Brown S, Findlay J, Amyes SGB. (2007) Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*. *Clinical Microbiology and Infection.* **13**: 1137-1138

Schneiders T, Findlay J, Amyes SGB. (2008) Efflux pumps *in Acinetobacter baumannii*. In Bergogne-Bérézin, E, Friedman H, Bendinelli M (Ed.) *Acinetobacter biology and pathogenesis.* (p105-127) Springer

Hamouda A, Findlay J, Al-Hassan L, Amyes SGB. (2010) The epidemiology of *Acinetobacter baumannii* of animal origin. *8th International Symposium on the Biology of Acinetobacter,* Rome, Italy (1-3rd September) Abstract no. P33

Lopes BS, Hamouda A, Findlay J, Amyes SGB. (2011) The effect of frame-shift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*. *Journal of Medical Microbiology.* **60**: 211-215

Hamouda A, Findlay J, Amyes SGB. (2011) Carbapenems: do they have a future? *Journal of Medical Microbiology.* **60**: 1229-1230

Hamouda A, Findlay J, Al-Hassan L, Amyes SGB. (2011) The epidemiology of *Acinetobacter baumannii* of animal origin. *International Journal of Antimicrobial Agents*. **38**: 314-318

VI. Abbreviations.

ABC	ATP binding cassette
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	Base pairs
BSAC	British Society For Antimicrobial Chemotherapy
BSI	Bloodstream infection
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CDC	Center For Disease Control
cfu	Colony forming units
Cip	Ciprofloxacin
Cm	Chloramphenicol
CPS	Capsular polysaccharide
CPZ	Chlorpromazine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
EPI	Efflux pump inhibitor
ETP	Ertapenem
FIC	Fractional inhibitory concentration
HAI	Hospital acquired infection
HPA	Health Protection Agency
HTH	Helix-turn-helix
ICU	Intensive care unit
IEF	Iso-electric focussing
IPM	Imipenem
IST	Iso-sensitest
kb	Kilo-base pairs
kDa	Kilo-Dalton
KPC	*Klebsiella pneumoniae* carbapenemase

LB	Luria-Bertani
LPS	Lipopolysaccharide
M	Molar
MATE	Multidrug and toxic compound extrusion
mA	Milli-amps
MDR	Multidrug resistant
MEM	Meropenem
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
ml	Milli-litre
mm	Milli-metre
mM	Milli-molar
mRNA	Messanger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Centre of Biotechnology Information
NDM	New Delhi Metallo-β-lactamase
NHS	National Health Service
Nor	Norfloxacin
OD	Optical density
OMP	Outer membrane protein
ORF	Open reading frame
PABN	Phenyl alanine arginyl b-naphtylamide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDR	Pandrug resistant
PFGE	Pulsed-field gel electrophoresis
pH	Measure of the acidity or basicity of a solution
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RND	Resistance nodulation division

RTI	Respiratory tract infection
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SMR	Small multi-drug resistance
SSI	Surgical site infection
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBE	Tris-borate-ethylenediaminetetraacetic acid
TE	Tris-EDTA
Tet	Tetracycline
Tige	Tigecycline
TMS	Transmembrane segment
UTI	Urinary tract infection
V	Volts
W	Watts
w/v	Weight by volume
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar

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Chapter 1: Introduction

1.1 A brief history of antibiotics

1.1.1 Definition of antibiotics

Traditionally antibiotics are defined as natural microbial products that can kill or inhibit the growth of other micro-organisms, however synthetically produced antibiotics are now also referred to under the same heading.

1.1.2 The emergence of antibiotics

Antibiotics were introduced into medicine over 70 years ago, significantly altering life expectancy (Lesche, 2007). Antibiotics have enabled the successful treatment of previously life-threatening infections and allowed for the use of interventions, such as surgery, for medical conditions which were previously thought to be untreatable due to the high risks of infection. The first antibiotics, the sulphonamides (Prontosil®), were put into commercial use in the 1930's (reviewed by Lesche, 2007). Prontosil® was a man-made antibiotic with broad antimicrobial activity against Gram-positive bacteria but with no effect on Enterobacteriaceae (reviewed by Lesche, 2007). This was quickly followed by other sulphonamide drugs prior to the use of the first natural antibiotic, penicillin, a β-lactam class antibiotic derived from the mould *Penicillium chrysogenum*, the discovery of which was accredited to Alexander Fleming in 1928 (reviewed by Quirke, 2001). Despite its early discovery,

penicillin was not commercially available for clinical use until the early 1950's due to difficulties in its purification and production (reviewed by Quirke, 2001). In the years following their initial discoveries and availability for clinical utilisation, antibiotic use rose exponentially and several new classes of both man-made and naturally occurring antibiotics were discovered including the aminoglycosides in 1943 and tetracycline in 1955. By the end of the 1960's the development of new antibiotics had all but stopped and pharmaceutical companies instead began altering the molecular structure of already existing antibiotics in order to overcome bacterial resistance mechanisms and toxicity issues (Schlaes *et al*, 2004). It was not until the late 1990's and early 2000's that any further new antibiotic classes were discovered and made available for clinical use; the lipopeptides (daptomycin), the glycylcyclines (tigecycline) and the oxazolidinones (linezolid). Currently new antibiotics are scarce with few compounds making it through safety and efficacy trials to commercial production.

1.2 Antibiotic classes

1.2.1 Antibiotic mechanisms of action

Antibiotics can exert their antimicrobial effects via five main mechanisms; inhibition of DNA synthesis, inhibition of RNA synthesis, inhibition of protein synthesis, inhibition of cell wall synthesis, and inhibition of tetrahydrofolate synthesis.

Inhibition of DNA synthesis

Example: The fluoroquinolones target DNA gyrase and topoisomerase IV, preventing the unwinding of supercoiled DNA and therefore inhibiting DNA transcription and replication.

Inhibition of RNA synthesis

Example: Rifampicin binds the β-subunit of RNA polymerase, preventing RNA transcription and any subsequent translation.

Inhibition of protein synthesis

Example: Tetracyclines bind to the 16S part of the 30S ribosomal RNA subunit preventing the binding of amino-acyl tRNA and inhibiting translation.

Inhibition of cell wall synthesis

Example: β-lactam antibiotics inhibit transpeptidase and peptidoglycan synthesis preventing the cross linking of the polysaccharide chains in the bacterial cell wall.

Inhibition of tetrahydrofolate synthesis

Example: The sulphonamides competitively inhibit dihydropteroate synthetase resulting in the disruption of the tetrahydrofolate synthase pathway, which is essential for folate biosynthesis.

Antibiotics are typically classed as either bactericidal or bacteriostatic towards their targets, however some can act in either respect, usually dependent upon the concentration of antibiotic used and the target bacterium. Bactericidal antibiotics are capable of killing the bacteria whilst bacteriostatic antibiotics inhibit bacterial reproduction. Table 1.1 shows the mechanisms by which each class of antibiotic exerts its effects.

Mechanism of Action	Antibiotic Class	Sub-Class	Bacteriostatic/Bactericidal	Examples
Inhibition of DNA synthesis	Fluoroquinolones		Bactericidal	Ciprofloxacin, Norfloxacin
	Metronidazole		Bactericidal	Metronidazole
	Quinolones		Bactericidal	Nalidixic acid
Inhibition of RNA synthesis	Rifampicin		Bactericidal	Rifampicin
Inhibition of protein synthesis	Aminoglycosides		Bactericidal	Amikacin, Gentamicin
	Chloramphenicols		Bacteriostatic	Chloramphenicol, Thiamphenicol
	Glycylcyclines		Bacteriostatic	Tigecycline
	Lincomycins		Bacteriostatic	Clindamycin, Lincomycin
	Macrolides		Bacteriostatic	Azithromycin, Erythromycin
	Oxazolidinones		Bacteriostatic	Linezolid
	Tetracyclines		Bacteriostatic	Minocycline, Tetracycline
Inhibition of cell wall synthesis	β-lactams	Carbacephems	Bactericidal	Loracarbacef
		Carbapenems		Ertapenem, Meropenem
		Cephalosporins - 1st Gen		Cefazolin, Cefalexin
		2nd Gen		Cefoxitin, Cefuroxime
		3rd Gen		Cefotaxime, Cefpodoxime
		4th Gen		Cefepime
		5th Gen		Ceftobiprole
		Monobactams		Aztreonam
		Penicillins		Amoxicillin, Cloxacillin
	Fosfomycin		Bactericidal	Fosfomycin
	Glycopeptides		Bactericidal	Teicoplanin, Vancomycin
	Lipopeptides		Bactericidal	Daptomycin
	Polypeptides		Bactericidal	Bacitracin, Colistin
Inhibition of tetrahydrofolate synthesis	Diaminopyrimidines		Bacteriostatic	Iclaprim, Trimethoprim
	Sulphonamides		Bacteriostatic	Sulphadiazine, Sulphamethoxazole

Table 1.1. **Classes of antibiotics.** The antibiotic classes and their mechanisms of action.

1.2.2 Synergistic effects of antibiotic combinations

In cases of difficult or highly resistant infections combinations of antibiotics, usually of different mechanisms of action or antibiotics which show synergy together, can be used to eliminate such infections. Antibiotic synergy is defined as when the effects of the combination of two or more antibiotics is greater than the sum of the effects of the individual antibiotics (Gould *et al*, 1991).

The aminoglycosides and β-lactams are two antibiotic classes with different mechanisms of action that can be more active when used together than separately (Gavalda *et al*, 2003). Aminoglycosides are bactericidal protein synthesis inhibitors and are required to penetrate the bacterial cell and bind the 30S ribosome to exert their antimicrobial action. The β-lactams are bactericidal cell wall inhibitors which bind to the penicillin binding proteins to inhibit peptidoglycan synthesis. When used together, the action of the β-lactams inceases the permeability of the cell wall allowing a higher rate of penetration for the aminoglycosides, and a synergistic effect can be observed.

Another common example of this is the use of clavulanic acid with cephalosporins. Clavulanic acid on its own shows little antimicrobial activity, however it is capable of competitively inhibiting some β-lactamase enzymes due to its possession of a β-lactam ring structure similar to that found in the β-lactam class antibiotics. This can allow a β-lactam antibiotic to exert antimicrobial effects against the bacterium which it otherwise could not when used on its own.

One of the first observed examples of synergy was that of the sulphonamide, sulfamethoxazole, and the dihydrofolate reductase inhibitor, trimethoprim. The synergy between these two antibiotics was first observed in both *in vivo* and *in vitro* experiments in the 1960's (Bushby and Hitchings, 1968). Both antibiotics inhibit the tetrahydrofolate synthesis pathway, albeit at different steps. Sulfmethoxazole competitively inhibits dihydropteroate synthetase, the enzyme responsible for dihydropteroic acid synthesis and trimethoprim inhibits the conversion of dihydrofolic acid into the end-product, tetrahydrofolic acid. This combination is available as co-trimoxazole which contains a 1:5 ratio of trimethoprim to sulfamethoxazole. Co-trimoxazole shows activity in bacterial, protozoan and fungal infections, and consequently is often used as a prophylactic agent in immune compromised (eg HIV) patients (Walker *et al*, 2010).

There are numerous other antibiotic combinations used clinically, even more so due to the ever increasing incidences of multidrug resistant (MDR) infections. It has been suggested that combination therapy can also limit the development of resistance and this tactic has long since been utilised in the treatment of infections such as tuberculosis (Crofton, 1960; Young, 2009).

1.3 The early development of antibiotic resistance

1.3.1 The end of bacterial infections?

Following the antibiotic boom of the 1950's and 1960's many experts had begun to believe that bacterial infections were to become a problem of the past. In 1969 the US Surgeon General, William Stewart, announced to the US congress that it was time to "close the books on infectious diseases" (Martin, 2008). However, this opinion was not shared with all in the scientific and medical community. Alexander Fleming had previously been quoted by the New York Times in 1945 saying, "The greatest possibility of evil in self-medication is the use of too small doses so that instead of clearing up infection the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and from then to others until they reach someone who gets a septicaemia or pneumonia which penicillin cannot save". This statement could now be considered to have been a fairly accurate prediction of the development of antibiotic resistance.

1.3.2 The development of resistance

The term antibiotic resistant is usually used to refer to an infection causing bacterium that cannot be successfully treated with an antibiotic at clinically achievable concentrations. Susceptibility can be defined as a minimum inhibitory concentration (MIC) that is equal to or less than the lowest MIC observed for the general population.

Bacteria were undoubtedly exhibiting resistance to antibiotics long before humans utilised antibiotics for medicinal purposes, largely due to most antibiotic classes being produced by other micro-organisms in the environment where bacteria would encounter them naturally and subsequently develop resistance mechanisms as a means of survival. The classic example of this is penicillin, a product of the fungus *P. chrysogenum* commonly found in soil, a habitat shared with several clinically relevant species of bacteria including *P. aeruginosa* and *Acinetobacter* spp. As a result of their natural habitat these organisms tend be intrinsically resistant to penicillin due to the possession of chromosomal β-lactamases (Jacoby, 2009).

Since the first discovery and utilisation of antibiotics in medicine in the 1930's we have witnessed what can be described as a dual evolution. On one hand there has been an increase in the number of antibiotic classes, their variants and spectrum of activity; whilst on the other hand there has been the emergence and dissemination of antibiotic resistance. It is unknown which resistance phenotypes were pre-existing and which developed as a consequence of antibiotic utilisation in humans. The effect on microbial populations following the first introduction of antibiotics cannot be determined and it is possible that some bacterial species were even completely eliminated as a result (Lesche, 2007). When the first wave of resistance was detected (to the penicillins), the introduction of newer antimicrobials were used to counteract the effect. This pattern of resistance development continued for almost all newly introduced antibiotics but was not disastrous for medicine due to the continuous discovery of newer alternatives. However, in recent years the rate of new antibiotic discovery has almost halted whilst the development of resistance appears to be

evolving faster than ever, and consequently we are witnessing the increasing emergence of MDR and pandrug resistant (PDR) strains (Levy and Marshall, 2004; Falagas and Bliziotis, 2007). As a result, treatment options are fast becoming limited and the quest for new antibiotics is of increasing importance.

It should also be stated that despite the emergence of antibiotic resistant strains, not all species of bacteria are suited to attaining an MDR phenotype upon exposure to antibiotics. The potential for the development of multidrug resistance is species-specific. For example *Streptococcus pyogenes* infections are commonly treated with penicillin antibiotics and yet *S. pyogenes* generally remains susceptible to the penicillins and other β-lactam antibiotics (Albrich *et al*, 2004).

1.4 Mechanisms of resistance

1.4.1 Resistance acquisition in bacteria

Bacteria typically acquire resistance through the following: mutations and the acquisition of genes. There are several mechanisms by which bacteria can develop resistance including: reduced permeability, active efflux, inactivation of the antibiotic via destruction or alteration, alteration of drug target, target amplification, and additional targets; all of which can be as a result of either resistance acquisition mechanism (Mazel and Davies, 1999).

1.4.2 Mobile genetic elements

There are two main categories of mobile genetic elements; those that are capable of horizontal transfer (moving from one cell to another), namely plasmids and bacteriophages, and those that can move from one genetic location to another within the same cell, transposons and integrons (Bennett, 2008). Mobile genetic elements have been linked to the dissemination of resistance genes to several antibiotic classes including the fluoroquinolones, aminoglycosides and β-lactams (Bennett, 2008).

1.4.2.1 Bacteriophages

Bacteriophages, also known as phages, are viruses that infect bacteria. Phages can undergo two types of replication, lytic or lysogenic. Phages are capable of carrying and disseminating antibiotic resistance genes by transduction. Lytic phages, such as T4 phage, penetrate the bacterial cell and replicate using the cells own replication machinery. This results in the immediate phage-mediated lysis of the bacterial cell wall and the release of the new phages which can then go on to infect new hosts. Lysogenic phages will penetrate the bacterial cell and incorporate their DNA into the host chromosomal DNA. The phage DNA will then replicate alongside the host DNA but cause no harm to the host. The virus lies dormant and continues to allow the host to replicate as normal, producing a copy of the virus DNA in each of the daughter cells. Bacteriophages are also capable of carrying plasmids, transposons and integrons, and so can facilitate their dissemination in this manner

1.4.2.2 Transposons

Transposons, also known as 'jumping genes', are mobile genetic elements that can move around within a genome by transposition. These sequences of DNA, found in both Gram-positive and Gram-negative bacteria, contain transposase genes and are identified by the presence of inverted repeated sequences at both ends of the element. In bacteria, transposons are capable of transposition between plasmid and chromosomal DNA and vice versa, often carrying genes encoding antibiotic resistance. Transposons are an essential mechanism for the dissemination of antibiotic resistance genes amongst bacteria. Of note, transposons that lack any additional genes are known as insertion sequences.

1.4.2.3 Integrons

Integrons are genetic elements that contain an integrase gene which mediates the integration of external DNA into the integron. They can 'poach' external chromosomal or plasmid DNA, including antibiotic resistance genes, and incorporate the DNA into the integron. Integrons often reside within transposons and so represent a mechanism by which new antibiotic resistance genes are added to the complement of a transposon and are subsequently disseminated.

1.4.2.4 Plasmids

Plasmids are self-replicating, circular DNA molecules that are capable of carrying a number of genes. Bacterial plasmids can range in size from 1kb to >1000kb (Finan *et al*, 2001). Bacterial plasmids act as a scaffold upon which gene arrays can be built, often encoding antibiotic resistance genes, by the incorporation of transposable elements and integron gene cassettes. A resistance plasmid is one which encodes resistance genes to one or more antibiotics (Bennett, 2008). Plasmids can provide a means by which bacterial species can dip into the resistance gene pool when necessary for their survival. Some plasmids have a particularly broad host range, for example the resistance plasmid RP1, initially identified in *P. aeruginosa,* is capable of dissemination amongst most Gram-negative species of bacteria (Bennett, 2008). Genes encoding resistance to several families of antibiotics, particularly the β-lactams, are commonly disseminated in this manner.

1.4.3 Mutations

The types of mutations resulting in resistance to antibiotics are wide and varied. For example, the target site for the antibiotic may be mutated so that the antibiotic can no longer bind and exert its effects or alternatively, mutations can occur in the regulation genes of efflux pumps causing them to be over-expressed. The mutations themselves may simply consist of single amino acid changes that alter the gene transcription, translation or efficacy of a particular protein, or alternatively may consist of insertions or deletions.

Mutations often occur as a direct result of antibiotic pressure, ie an adaptive response enabling the survival of the bacterium. In such cases the mutations are targeted towards particular genes and many of these have been well characterised in bacteria. For example, mutations within the *gyrA* gene have been associated with fluoroquinolone resistance in members of the Enterobacteriaceae (Weigel *et al*, 1998).

However, the effect of spontaneous mutations, in the absence of antibiotic pressure, cannot be underestimated. In *Mycobacterium tuberculosis*, it is a succession of such mutations that are thought to be responsible for the development of multidrug resistance rather any gene acquisition (Musser, 1995). An example of this is resistance to streptomycin in the absence of any aminoglycoside modifying enzymes which is instead thought to be caused by mutations in the 16S ribosomal RNA genes to which streptomycin usually binds (Musser, 1995).

1.4.4 Bacterial efflux pumps

Efflux pumps are proteins involved in the transport of toxic substrates across the cell membrane and into the external environment. These pumps are found extensively in both prokaryotic and eukaryotic organisms. Efflux pumps are present in all studied bacterial genomes and so can be considered to be essential for cell survival (Webber and Piddock, 2003). Such pumps are normally encoded within the bacterial chromosome but can also be found on plasmids, suggesting that they are capable of dissemination (Webber and Piddock, 2003). Small amphiphilic molecules, which

include members of several antibiotic families, can easily penetrate the bacterial cell membrane and so it is essential that the cell must have a mechanism with which to extrude unwanted and toxic compounds that may enter from the external environment, as well as those created within the cell by biological processes (Bambeke *et al*, 2000). The pumps can be specific for a particular substrate or alternatively they can have a broad substrate range. Efflux is an important mechanism of antibiotic resistance for two reasons: they allow the bacterium to cope and survive in a stressful environment (in the presence of antibiotics) and concurrently, by delaying the death of the bacterium they increase exposure time to the antibiotics in which the bacterium may undergo mutations in order to achieve higher levels of resistance.

Efflux pumps are increasingly implicated in the causes of antibiotic resistance within numerous clinically relevant bacterial species including *K. pneumoniae* and *P. aeruginosa* (Webber and Piddock, 2003; Yang *et al*, 2003). The presence of efflux is often gauged *in vitro* by the use of efflux pump inhibitors (EPIs) such as reserpine, CCCP and PABN. These are used alongside the relevant antibiotics to result in increased susceptibilities, when compared to the antibiotic alone, in the presence of active efflux. However EPIs are only used to gauge the presence of efflux in the laboratory and cannot be used clinically for treatment due to their toxicity and potential to interfere with other cellular functions (Garvey and Piddock, 2008). The presence of active efflux therefore presents a major problem in successfully treating infections with the usual first line antibiotics and such infections may require alternative drugs that are not subject to efflux.

There are several families of efflux pumps found in bacteria which are classified by three main criteria; their structural homology, substrate specificity, and energy source.

1.4.5 Efflux pump families

- The ATP binding cassette superfamily (ABC)
- The multidrug and toxic compound extrusion family (MATE)
- The major facilitator superfamily (MFS)
- The resistance nodulation cell division superfamily (RND)
- The small multidrug resistance family (SMR)

Table 1.2 summarises the properties of each efflux pump family.

Family/ Superfamily	Gram +ve/-ve	Example	Energy Source	TMS	Primary/ Secondary	References
ABC	+ve/-ve	LmrA, *L. lactis*	ATP	12	P	Bambeke *et al*, 2000.
MATE	-ve	YdhE, *E. coli*	PMF	12	S	Borges-Walmsley *et al*, 2003.
MFS	+ve/-ve	NorA, *S. aureus*	PMF	12 or 14	S	Yin *et al*, 2000.
RND	-ve	AcrAB, *E. coli*	PMF	12	S	Yang *et al*, 2003.
SMR	+ve/-ve	QacC, *S. aureus*	PMF	4	S	Leelaporn *et al*, 1994.

Table 1.2. **Bacterial efflux pump families.** A summary of the types of efflux pumps found in bacteria with structural information and examples. PMF – proton motive force, ATP – adenosine triphosphate, TMS – transmembrane segments.

In Gram-negative bacteria there are structural similarities between the RND, ABC and MFS pumps. All three are proposed to function with a membrane fusion protein (MFP) and outer membrane protein (OMP) (Paulsen *et al*, 1997). Whilst the MFP component of the pump is often exclusive to the transporter protein (eg AcrAB, MexAB), the OMPs have been shown to be somewhat promiscuous (Welch *et al*, 2010). It has been shown that TolC in *E. coli* can function alongside a number of different RND transporters as well as MFS transporters in the transport of iron (Bleuel *et al*, 2005).

1.4.5.1 ABC

The ABC transporter family forms one of the largest protein families, within both eurkaryotes and prokaryotes, with a broad substrate range including the uptake of sugars and the extrusion of toxic substances (Borges-Walmsley *et al*, 2003). These primary transporter systems are distinct from other efflux systems in that they source their energy from ATP hydrolysis for both the uptake and efflux of compounds/molecules. The pumps themselves constitute two transmembrane domains, each containing six helices, and two cytoplasmic domains. These pumps can function as either homodimers or heterodimers. LmrA is an example of a well characterised member of this superfamily which has been shown to be involved in conferring multidrug resistance in *Lactococcus lactis* (Bambeke *et al*, 2000).

1.4.5.2 MATE

MATE transporters comprise of a protein of approx. 450 amino acids in length arranged into 12 TMS. These transporters have been implicated in multidrug resistance in a number of bacteria as well as yeast (Kuroda and Tsuchiya, 2009). YdhE has been shown to confer resistance to cationic antimicrobials in *E. coli* (Borges-Walmsley *et al*, 2003).

1.4.5.3 MFS

The MFS efflux pumps are similar in structure to the RND family in that they typically have 12 or 14 TMS and require the use of an OMP component to complete their function. These pumps have been shown to be involved in the mediation of drug resistance but also in the uptake of various substrates such as sugars into the cell (Yin *et al*, 2000). The TetA(B) protein is an example of a well characterised member of this family which has been shown to transport tetracycline in *E. coli* (Yin *et al*, 2000).

1.4.5.4 RND

Of the five families of efflux pumps, the RND pumps are found most extensively and abundantly in Gram-negative bacterial species. These pumps have a broad substrate range and are capable of the efflux of several families of antibiotics. The RND efflux pumps are tripartite systems consisting of an OMP, a MFP and RND pump

protein consisting of 12 TMS (Figure 1.1). Many of the RND family pumps exist as part of an operon containing a control mechanism, usually a repressor gene, often shortly upstream of the pump encoding genes. The efflux genes are transcribed when the repressor is deactivated, most commonly by mutations. The ability of these pumps to be switched on when the bacterium is under antibiotic stress and their broad substrate range can lead to cross-resistance of a bacterium to several structurally unrelated antibiotics that they may not have previously encountered. The AcrAB pump, found in several members of Enterobacteriaceae, has been particularly well characterised in *E. coli* and shown to be capable of conferring an MDR phenotype upon its over-expression (Yang *et al*, 2003).

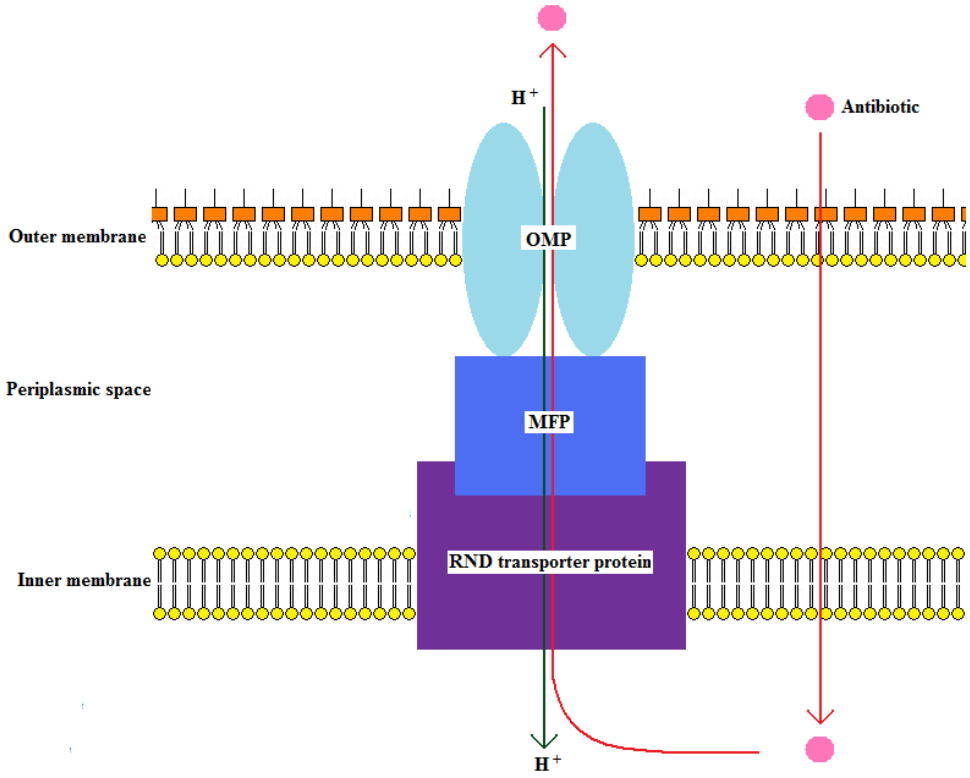

Figure 1.1. **RND family efflux pump.** The typical structure of an RND family efflux pump found in Gram-negative bacteria. (adapted from Schneiders *et al*, 2009)

1.4.5.5 SMR

SMR efflux pumps are relatively small comprising of around 100 amino acids which are arranged into four transmembrane helices. Despite their small monomeric size, SMR pumps are thought to function as trimers, making for a complex tertiary structure similar to that of the RND or MFS pumps. The QacC proteins found in staphylococcal species have been shown to confer resistance to several types of antiseptics and disinfectants (Leelaporn *et al*, 1994).

1.5 The bacterial outer membrane

1.5.1 Permeability of the outer membrane

The Gram-negative bacterial membrane is made up of a bilayer consisting of phospholipids, lipopolysaccharides and OMPs (Domenech-Sanchez *et al*, 1999). The membrane has several functions including acting as a barrier between the intracellular and extracellular environment and controlling the passage of molecules in and out of the cell. The porins, an OMP family of proteins, generally consist of two subgroups; specific, eg OprD in *P. aeruginosa*, and non-specific porins, eg OmpC and OmpF in *E. coli* (Domenech-Sanchez *et al*, 1999). Non-specific porins generally allow the diffusion of small polar molecules whilst specific porins only allow the diffusion of specific substrates (Domenech-Sanchez *et al*, 1999).

1.5.2 Major OMPs

A major cause or contributing factor to an antibiotic resistant phenotype in bacteria, particularly Gram-negative species, is the alteration of outer membrane permeability. In a number of members of the Enterobacteriaceae, including *E. coli*, *K. pneumoniae* and *Salmonella* spp., there are three major OMPs which were initially characterised and named in *E. coli* as OmpA, OmpC and OmpF. The primary function of these porins is to allow the passage of nutrients and other molecules in and out of the cell. This inadvertently allows the passage of several types of antibiotics. The loss of one or more of these porins can hinder or limit the ability of the antibiotic to enter the cell

and lead to a reduction in susceptibility. When in combination with active efflux, high levels of resistance to multiple antibiotics can be achieved. In *K. pneumoniae* the loss of the OmpF homologue OmpK35 has been associated with cefoxitin resistance (Ananthan and Subha, 2005). Porin loss is often due to mutations, insertions or deletions in the open reading frame or the promoter region of the encoding gene.

1.5.3 The major OMPs of *K. pneumoniae*

There are three major OMPs found in *K. pneumoniae*; OmpK34 (OmpA), OmpK35 (OmpF) and OmpK36 (OmpC). (*E. coli* homologues shown in brackets)

1.5.3.1 OmpK34

OmpK34 is a ~34kDa, monomeric, heat modifiable protein that forms a non-specific porin (Nikaido, 2003). Unlike the other major porins, OmpK34 is not reported to play any role in antibiotic resistance. Studies have shown in the OmpK34 homologues, OmpA from *E. coli* and OprF from *P. aeruginosa*, that these proteins form 'closed' porins that prevent the permeation of large molecules such as antibiotics (Nikaido, 2003). Comparisons with OmpC and OmpF have shown that solutes permeate OmpA at a rate two orders of magnitudes lower (Nikaido, 2003). OmpA has been indicated to be integral for the stability of the outer membrane and play a role in F-mediated conjugation (Riede and Eschbach, 1986).

1.5.3.2 OmpK35

OmpK35 is a ~40kDa, trimeric protein that forms a non-specific porin (Lee *et al*, 2006). OmpK35 has been reported to allow the penetration of several β-lactam antibiotics including cefotaxime, cefoxitin, and to a lesser extent imipenem and meropenem (Domenech-Sanchez *et al*, 1999). OmpK35 is reported to allow the passage of slightly larger solutes than OmpK36 with a preference for cations over anions (Nikaido, 2003). A study has suggested that clinical exposure to cefuroxime can deplete the expression of OmpK35 (Kallmann *et al*, 2008). OmpK35 is classed as an osmoporin as evident by its reduced expression in a high osmolarity environment (Dutzler *et al*, 1999).

1.5.3.3 OmpK36

OmpK36 is a ~38kDa, trimeric protein that forms a non-specific porin. Examination of the diffusion rates of organic molecules through the homologous porins OmpC and OmpF suggest that OmpC is a slightly smaller porin (Nikaido, 2003). The loss of OmpK36 has been associated with resistance to the β-lactams, including the carbapenems. It has been shown that in *K. pneumoniae* strains exhibiting resistance to carbapenems, OmpK36 is often absent, suggesting that the carbapenem antibiotics are capable of permeating the cell through this porin (Kaczmarek *et al*, 2006).

1.6 The β-lactam antibiotics

1.6.1 The β-lactam class

The β-lactams are a family of antibiotics that are characterised by the presence of a β-lactam ring (Figure 1.2). They are a diverse and varied family which include the penicillins, cephalosporins and carbapenems, and are the most commonly prescribed antibiotics in Europe (Molstad *et al*, 2002). Collectively β-lactams show activity against Gram-negative and Gram-positives organisms, including anaerobes. The penicillins, despite being one of the first discovered antibiotics, remain one of the most commonly prescribed antibiotics, particularly for urinary tract infections (UTIs), largely due to their high absorption rates (Holten and Onsuko, 2000). For more complicated or resistant infections, the cephalopsorins are often prescribed due to their broader spectrum of activity.

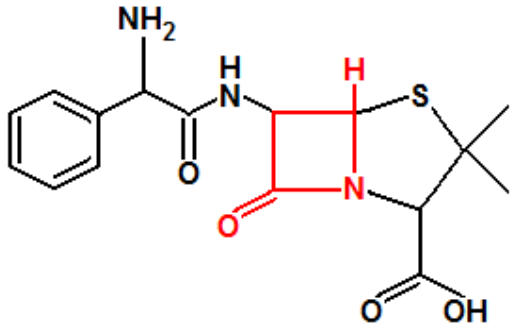

Figure 1.2. **Primary structure of ampicillin.** The primary structure of the β-lactam antibiotic ampicillin. The β-lactam ring is highlighted in red. (adapted from Quirke, 2001)

The ever-increasing use of β-lactam antibiotics has resulted in the inevitable development of resistance, most of which is due to the production and dissemination of β-lactamases. β-lactam resistance is widespread amongst bacteria and a considerable and continually growing number of β-lactamases have now been identified and characterised. Two approaches have been used to overcome this resistance; the development of new β-lactams that are not degraded/hydrolysed by β-lactamases, and the use of β-lactamase inhibitors, such as clavulanic acid, sulbactam or tazobactam, which work in synergy with the β-lactam antibiotics.

1.6.2 The carbapenems

Carbapenems are considered to be the most powerful of the β-lactam antibiotics and are often used as a last resort drug to treat infections that are resistant to the cephalosporins. There are four members of the carbapenems that are used clinically: doripenem, ertapenem, imipenem and meropenem.

1.6.2.1 Doripenem

Doripenem is the most recent of the carbapenems licensed for clinical use in the UK in 2008 for the treatment of complicated intra-abdominal infections, nosocomial pneumonia and ventilator associated pneumonia (European Medicines Agency, 2008). It is a broad spectrum, synthetic carbapenem antibiotic reported to be β-lactam stable and resistant to inactivation by renal enzymes. Doripenem has been shown to exhibit comparable activity to imipenem and meropenem against both

Gram-positive and Gram-negative organisms, but poorer activity against anaerobic organisms (Greer, 2008). Despite being licensed, the few reported advantages over imipenem and meropenem, coupled with the increased costs have meant that this antibiotic is still rarely used compared with the other carbapenems (Greer, 2008).

1.6.2.2 Ertapenem

Ertapenem is a once daily parenteral 1-β-methyl carbapenem antibiotic licensed in 2002 for use against intra-abdominal, gynaecological and community acquired pneumonia (Livermore *et al*, 2003). Ertapenem has a more limited spectrum of activity, showing poor activity particularly against non-fermenting bacteria (eg *Pseudomonas* spp.), in respect to the other carbapenems and is largely used for the treatment of community acquired infections, particularly those caused by extended-spectrum β-lactamase (ESBL) carrying bacteria (Livermore *et al*, 2003).

1.6.2.3 Imipenem

Imipenem was developed in 1985 from a naturally produced antibiotic, Thienamycin, from *Streptomyces cattleya* which itself showed broad spectrum activity against Gram-positive, Gram-negative and anaerobic organisms (Kahan *et al*, 1983; Merck, 2010). Unfortunately thienamycin instability meant that it was impractical for the clinical treatment of bacterial infections and instead a derivative of thienamycin, imipenem, was developed and synthesised. Due to its instability against renal enzymes, imipenem must be administered with cilastatin, a dehydropeptidase

inhibitor, in order to prevent its degradation (Merck, 2010). Imipenem is indicated for the treatment of serious and complicated infections of the respiratory tract, skin as well as gynaecological and intra-abdominal infections (Merck, 2010). Compared with the other carbapenems, imipenem exhibits increased toxicity in adults with impaired renal function and as such requires careful monitoring, particularly in elderly patients (Merck, 2010).

1.6.2.4 Meropenem

Meropenem is a broad spectrum, synthetic carbapenem, licensed for use in the UK in 1988. Similarly to imipenem, meropenem exhibits broad activity against Gram-positive, Gram-negative and anaerobic organisms; however unlike imipenem, structural differences in meropenem mean it is stable against degradation by dehydropeptidase. Meropenem is indicated for the treatment of serious and complicated infections of the skin as well as intra-abdominal infections and bacterial meningitis (Astra Zeneca, 2009).

1.7 β-lactamases and extended-spectrum β-lactamases (ESBL's)

1.7.1 β-lactamases

β-lactamases are enzymes produced by some species of bacteria that are capable of conferring resistance to β-lactam antibiotics via the hydrolysis of the β-lactam ring. β-lactamases can be split into two main groups; those that contain a serine residue at

their active site, and those that require the metal ion zinc as a co-factor, known as metallo-β-lactamases (Jacoby and Munoz-Price, 2005). β-lactamases can also be grouped into four molecular classes, A to D, based on their primary structure. Classes A, C, and D contain a serine residue at their active site and class B comprises the metallo-β-lactamases (Jacoby and Munoz-Price, 2005). These molecular classes can be further split into functional groups based on their substrate spectrum and response to inhibitors (Table 1.3) (Bush *et al*, 1995).

Bush-Jacoby-Medeiros Functional Classification	Molecular Class	Preferred Substrates	Enzyme Type	Example
1	C	Cephalosporins not inhibited by CA*	Cephalosporinases	AmpC
2a	A	Penicillins	Penicillinases	Penicillinases from Gram-positive species
2b	A	Penicillins, Cephalosporins	Cephalosporinase	TEM-1
2be	A	Penicillins, narrow and broad spectrum cepalosporins, monobactams	Cephalosporinase	SHV-2
2br	A	Penicillins	Inhibitor resistant penicillinases	TEM-30
2c	A	Penicillins, carbenicillin	Carbenicillinase	PSE-1
2d	D	Penicillins, cloxacillin	Cloxacillinase	OXA-1
2e	A	Cephalosporins	Cephalosporinase	Inducible penicillinases from *Proteus vulgaris*
2f	A	Penicillins, cephalosporins, carbapenems	Carbapenemase	KPC
3	B	Most β-lactams	Metallo-β-lactamase	VIM
4	ND	Penicillins not inhibited by CA*	Penicillinases	Penicillinase from *Pseudomonas cepacia*

Table 1.3. **β-lactamase classification.** β-lactamases as grouped by their molecular class, functional classification and substrate preference. Table modified from Bush *et al*, 1995. * CA = clavulanic acid.

1.7.1.1 Chromosomal β-lactamases

One of the most common examples of chromosomal β-lactamases are encoded by *ampC* genes found in several members of the Enterobacteriaceae and other bacterial species including *P. aeruginosa* and *A. baumannii*. These class C β-lactamses can mediate resistance to the penicillins as well as some of the broad spectrum cephalosporins. AmpC enzymes have even been associated with resistance to the carbapenem antibiotics when over-expressed in conjunction outer membrane porin loss (Jacoby, 2009). Although they are found chromosomally in some species, *ampC* genes can additionally be plasmid encoded.

The OXA β-lactamases similarly to AmpC, can be either chromosomally located (eg, OXA-51 in *A. baumannii*) or plasmid encoded (OXA-1 in *E. coli*). These enzymes are narrow spectrum class D penicillinases although some of their derivatives are classed as ESBLs, some of which are capable of conferring resistance to the cephalosporin and carbapenem antibiotics.

1.7.1.2 Plasmid encoded β-lactamases

In the 1960's the first plasmid-mediated β-lactamase in Gram-negative bacteria was discovered in an *E. coli* strain in Greece. The enzyme was named TEM-1, after the patient, a Greek girl named Temoneira, from which it was isolated (reviewed by Blomberg *et al*, 2005). Another enzyme TEM-2 was discovered shortly afterwards and was found to differ from TEM-1 by a single amino acid (reviewed by Blomberg

et al, 2005). Both enzymes were shown to display similar properties in the hydrolysis of penicillins and the narrow spectrum cephalosporins, such as cefazolin. There are currently over 130 TEM variant enzymes, and still counting, based on their amino acid sequences that are disseminated widely amongst Enterobacteriaceae via the means of plasmids (Jacoby and Munoz-Price, 2005). Presently there are numerous other plasmid encoded β-lactamase enzymes types with dozens of variants including the SHV-1 enzyme, which similarly to the TEM-1 and TEM-2, enzymes have a narrow spectrum of activity and have become widely spread amongst members of the Enterobacteriaceae (Wu *et al*, 2001). Another example of plasmid bound β-lactamases are the OXA enzymes (molecular class D), found mainly in *Acinetobacter* spp. and *P. aeruginosa,* although some have also been found throughout members of the Enterobacteriaceae (Naas and Nordmann, 1999).

1.7.2 ESBL's

ESBL's were initially discovered in the 1980's, primarily within *Klebsiella* spp., and found in immune compromised patients. ESBLs can be considered the most significant mechanism of resistance to the oxyimino-cephalosporins, mediating resistance to the broad-spectrum cephalosporins including the 3rd generation cephalosporins. ESBLs are most commonly found on plasmids, usually alongside other resistance determinants (Paterson *et al*, 2003). ESBL carrying strains of *K. pneumoniae* are more likely to be resistant to the fluoroquinolones and aminoglycosides in comparison to non-ESBL carrying strains (Paterson *et al*, 2003). The majority of ESBLs are found within *Klebsiella* spp. and *E. coli*, but are also

found organisms such as *P. aeruginosa*, *Enterobacter* spp. and *Salmonella* spp. (Chaudhary and Aggarwal, 2004).

1.7.2.1 TEM and SHV

TEM and SHV enzymes share a 68% amino acid homology and have very similar tertiary structures (Majiduddin and Palzkill, 2003). The majority of ESBLs stem from the class A narrow spectrum β-lactamases TEM-1, TEM-2 and SHV-1, via a series of mutations that alter the active site of these enzymes. In the TEM enzymes for example, the mutations within the active site of the enzyme alter its conformation, allowing access to the oxyimino-β-lactams (Jacoby and Munoz-Price, 2005). Although these mutations allow the enzyme to exhibit a greater spectrum of activity against β-lactam antibiotics, the conformational change also results in the enzyme becoming susceptible to β-lactamase inhibitors such as clavulanic acid (Jacoby and Munoz-Price, 2005). Collectively there are currently over 300 TEM and SHV β-lactamase derivatives (www.lahey.org/studies/webt.asp) and this number is increasing constantly, indicative of their rapid rate of evolution, likely in response to exposure to cephalosporin antibiotics. Mutations at amino acid positions 39, 69, 104, 164, 179, 205, 237, 238, 240, 244, 265 and 276 have been found in TEM and SHV variants, and are associated with their conversion into an ESBL phenotype (Knox, 1995). The presence of both SHV and TEM enzymes is very common in *K. pneumoniae*, particularly in clinical isolates, and these enzymes represent a means by which strains can potentially become resistant to the cephalosporin antibiotics. It has been reported that the SHV enzymes are 'universal' in the *K. pneumoniae*

chromosome, although they can additionally be found on plasmids (Babini and Livermore, 2000).

1.7.2.2 CTX-M

CTX-M enzymes are ESBLs that are most commonly found in members of the Enterobacteriaceae, particularly *E. coli* and *K. pneumoniae*. Sequence homology suggests that they evolved from the chromosomal β-lactamases of *Kluyvera* spp. (Livermore and Hawkey, 2005). There are currently over 100 CTX-M variants which exhibit varying levels of resistance to the oxyimino-cephalosporins (www.lahey.org/studies/webt.asp.). The first CTX-M enzyme found in the UK was little over a decade ago in 2000, in a strain of *K. oxytoca*, soon followed by the first reported outbreak caused by *K. pneumoniae* (Livermore and Hawkey, 2005). CTX-M-15 is the predominant CTX-M variant in clinical isolates in the UK (Livermore *et al*, 2007). It has additionally been reported that some CTX-M enzymes may be capable of contributing to the conferring of resistance to the carbapenem antibiotic, ertapenem, although this remains to be substantiated (Girlich *et al*, 2009).

1.7.2.3 OXA

OXA genes are predominantly found within *Acinetobacter* spp. and *P. aeruginosa* and to a lesser extent in members of the Enterobacteriaceae including *E. coli* and *K. pneumoniae* (Naas and Nordmann, 1999). These molecular class D genes were named for their ability to hydrolyse and confer resistance to the penicillin, oxacillin.

Although the first discovered OXA genes, such as OXA-1, were only found to confer resistance to the penicillins and had no effect on other β-lactam antibiotics, ESBL OXA derivatives have arisen that are capable of conferring resistance to the cephalosporins and carbapenems (Naas and Nordmann, 1999). There are currently over 100 OXA gene derivatives, 16 of which are ESBLs derived from OXA-2 and OXA-10 (www.lahey.org/studies/webt.asp.). The OXA genes are mainly found on plasmids although in some cases, such as that of OXA-51 in *A. baumannii*, they can be chromosomal.

1.7.3 Carbapenemases

Carbapenemases are a subgroup of the β-lactamase enzymes that are considered to be the greatest cause for concern in Gram-negative bacteria. Carbapenemases are capable of hydrolysing not only the cephalosporins and oxyimino-cephalosporins but also the carbapenem antibiotics. The carbapenem antibiotics are often considered the last resort drugs for the treatment of complicated MDR infections caused by Gram-negative pathogens and the increasing presence of the carbapenemase enzymes endangers their viability as a therapeutic option. These genes are predominantly found in members of the Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp., and can be located on either the chromosome or on plasmids (Table 1.4). There are carbapenemase members within all four molecular classes of β-lactamases, examples of which are shown below.

Class A – the *Klebsiella pneumoniae* carbapenemase (KPC) enzymes were first described in *K. pneumoniae* in 2001 (Yigit *et al*, 2001) and have since been found in several members of the Enterobacteriaceae (LaBombardi, 2007).

Class B – metallo-carbapenemases such as the Verona integron-encoded metallo-β-lactamase (VIM) which was first discovered in a strain of *P. aeruginosa* in Italy in 1999 and is now found globally as several variants and in a number of different bacterial species (Lauretti *et al*, 1999).

Class C – CMY-10, first isolated in *Enterobacter aerogenes* in 2003, is a plasmid bound carbapenemase and one of only a few that belong to β-lactamase molecular class C (Lee *et al*, 2003).

Class D – members of the OXA-type (oxacillin hydrolysing) β-lactamases usually exhibit a limited spectrum of activity but extended activity has been observed against the oxyimino cephalosporins and carbapenems caused by OXA-2 and OXA-10 derivatives (Naas and Nordmann, 1999).

Gene	Class	Organism(s)	Location	References
GES	A	*P. aeruginosa*, Enterobacteriaceae	Plasmid	Queenan and Bush, 2007.
IMI	A	*E. cloacae*	Chr/Plasmid	Queenan and Bush, 2007.
KPC	A	*K. pneumoniae*, *E. coli*	Plasmid	Queenan and Bush, 2007.
NMC	A	*E. cloacae*	Chr	Queenan and Bush, 2007.
SFC-1	A	*S. fonticola*	Chr	Henriques *et al*, 2004.
SHV-38	A	*K. pneumoniae*	Chr	Poirel *et al*, 2003.
SME	A	*S. marcesens*	Chr	Queenan and Bush, 2007.
AIM	B	*P. aeruginosa*	Plasmid	Gupta, 2008.
CcrA	B	*B. fragilis*	Chr	Rasmussen *et al*, 1990.
DIM	B	*P. stutzeri*	Plasmid	Poirel *et al*, 2010.
GIM	B	*P. aeruginosa*	Plasmid	Castanheira *et al*, 2004.
IMP	B	Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter* spp.	Chr/Plasmid	Queenan and Bush, 2007.
KHM	B	*C. freundii*	Plasmid	Sekiguchi *et al*, 2008.
NDM	B	Enterobacteriaceae	Plasmid	Yong *et al,* 2009.
SIM	B	*Acinetobacter* spp.	Chr	Lee *et al*, 2005.
SPM	B	*P. aeruginosa*	Plasmid	Toleman *et al*, 2002.
VIM	B	*P. aeruginosa*, *Acinetobacter* spp.	Chr/Plasmid	Queenan and Bush, 2007.
CMY	C	Enterobacteriaceae	Plasmid	Lee *et al*, 2003.
OXA	D	*Acinetobacter* spp.	Chr/Plasmid	Naas and Nordmann, 1999.

Table 1.4. **Carbapenemases.** Carbapenemase genes, their molecular class, genetic location and the organisms in which they have been found.

1.8 Gene regulation

1.8.1 Bacterial transcriptional regulators

In the environment bacteria are constantly exposed to any number of changing conditions and potential stressors such as changes in temperature, exposure to toxic molecules, osmotic stress and nutrient limitations. In order to survive in such fluid environments, bacteria must be capable of quickly adapting via the implementation of the appropriate survival mechanisms. Such adaptations often require the immediate regulation of specific gene sets. Regulatory proteins are usually involved in the mediation of such responses in response to specific environmental and cellular signals (Ramos *et al*, 2005). Transcriptional regulators are proteins that, as their name suggests, are involved in the regulation of gene transcription as either activators, repressors or both (Martinez-Bueno *et al*, 2004). There are several families of transcriptional regulators found in bacteria, grouped by their sequence similarity, structure and function. Table 1.5 lists the major families of transcriptional regulators found in bacteria.

Regulator Family	Activator/ Repressor	Motif	Terminal Position	Example	Function	Reference
AraC/XylS	Activator	HTH	C	MarA	Involved in conferring an MDR phenotype via efflux regulation in *E. coli*.	Cohen *et al*, 1993.
ArsR	Repressor	HTH	Central	CzrA	Repressor of the *czr* operon in *Staphylococus aureus*, involved in the transport of zinc and cobalt.	Busenlehner *et al*, 2003.
AsnC	Both	HTH	N	AsnC	Activator of *asnA* in *E. coli,* involved in the synthesis of asparagine from aspartate.	Kolling and Lother, 1985.
Cold Shock	Activator	RNA binding domain	Variable	CspC	Activator of the cold shock response and involved in RpoS regulation.	Rath and Jawali, 2006.
Crp	Both	HTH	C	PrfA	Activator of virulence factors in *Listeria monocytogenes*.	Uhlich *et al*, 2006.
DeoR	Repressor	HTH	N	SugR	Repressor of *ptsG*, phosphoenolpyruvate dependent phophotransferase.	Engels and Wendisch, 2007.
GntR	Repressor	HTH	N	GntR	Repressor of gluconate metabolism genes *gntU* and *gntK*.	Tong *et al*, 1996.
IcIR	Both	HTH	N	IcIR	Repressor of the *aceBAK* operon in *E. coli*.	Molina-Henares *et al*, 2006.
LacI	Repressor	HTH	N	LacI	Repressor of the *lac* operon in *E. coli*.	Nguyen and Saier, 1995.
LuxR	Activator	HTH	C	LuxR	Activator of the *lux* system responsible for luminescence and polyhydroxybutyrate synthesis in *Vibrio harveyi*.	Miyamoto *et al*, 1998.
LysR	Both	HTH	N	OxyR	Activator of genes involved in oxidative stress in *E. coli*.	Maddocks and Oysten, 2008.
MarR	Both	HTH	Central	MarR	Repressor of MDR gene *marA* in *E. coli*.	Alekshun and Levy, 1999.
MerR	Both	HTH	N	SoxR	Activator of *soxS* in response to oxidative stress.	Brown *et al*, 2003.
NirC	Activator	HTH	C	NirC	Activator of genes involved in nitrogen uptake.	Clegg *et al*, 2002.
OmpR	Activator	Winged helix	C	OmpR	Regulator of porin expression.	Maedo and Mizuno, 1988.
TetR	Repressor	HTH	C	AcrR	Local repressor of *acrAB* efflux pump in *E. coli*.	Schneiders *et al*, 2003.

Table 1.5. **Bacterial regulator families.** The families of transcriptional regulators found in bacteria.

1.8.2 Function

Regulator family proteins that act as activators, such as members of the AraC/XylS family, commonly function via the enhancement of the interaction between RNA polymerase and the promoter of the target gene(s) thus increasing transcription. Those that act as repressors, such as members of the TetR family, commonly function via the binding and blocking of the promoter region of the target gene, thus preventing the binding of RNA polymerase and transcription (Ramos *et al*, 2005). Alternatively regulators, such as OmpR, can be part of a two-component regulatory system which consists of a membrane bound histidine kinase and a DNA binding protein that acts as a response regulator (Maeda and Mizuno, 1988). Upon the detection of the specific signal the DNA binding protein is phosphorylated resulting in a conformational change and the subsequent mediation of transcription of the target gene.

1.8.3 TetR family proteins

TetR family proteins are regulatory proteins that are widely disseminated in bacteria and archaea. Family members are characterised by a high degree of similarity over a 47-residue stretch containing the DNA binding domain at their N-terminal end (Ramos *et al*, 2005). The C-terminal of TetR proteins is typically not well conserved indicating that this is likely to be the region involved in binding to specific drug/molecule targets (Ramos *et al*, 2005). TetR regulators typically act as repressors and bind as homodimers to their targets (Ramos *et al*, 2005).

Characterised family members have been shown to be involved in the regulation of a number of cellular functions including multidrug resistance, virulence and various catabolic pathways (Ramos *et al*, 2005). TetR family members have been shown to be particularly abundant in soil micro-organisms, possibly due to the varied environments that such micro-organisms encounter (Ramos *et al*, 2005). In contrast, TetR family proteins are not found in intracellular bacteria, such as *Chlamydia,* likely as a consequence of their stable environments (Ramos *et al*, 2001). These observations suggest that many TetR family proteins are primarily involved in the regulation of genes that are required for rapid adaptive physiological responses to environmental stimuli. A typical example of a TetR family protein can be found in AcrR.

1.8.3.1 AcrR

AcrR is one of the most well characterised members of the TetR family. AcrR acts as the local repressor of the co-transcribed *acrAB*, an RND family efflux pump found in Enterobacteriaceae and associated with conferring resistance to several antibiotic classes. The *acrR* gene is located upstream of and divergently transcribed from *acrAB*. AcrR is thought to repress *acrAB* transcription by binding to the promoter region, blocking RNA polymerase access and preventing transcription. *acrAB* is derepressed upon the binding of a drug at the C-terminal end of AcrR resulting in a conformational change at the N-terminus and subsequent release of AcrR from the promoter DNA (Su *et al*, 2007). In the case of MDR bacterial strains of *K. pneumoniae*, mutations are commonly found within the *acrR* gene which result in its

non-function, and subsequently upregulation of efflux through the AcrAB pump (Schneiders *et al*, 2003; Olliver *et al*, 2004).

1.8.4 The AraC/XylS family

The AraC/XylS family of regulators are one of the most common families of regulators with over 100 members identified in both Gram-positive and Gram-negative bacteria, however to date no members have been found in archaea or in eukaryotes (Gallegos *et al*, 1997). Members are typically around 100-350 residues long and are characterised by significant homology in a 100 residue section of amino acids containing the DNA binding domain (Gallegos *et al*, 1997). Most family members are proposed to contain two α-helix-turn-α-helix (HTH) DNA binding motifs (Gallegos *et al*, 1997). Homology studies revealed that the first HTH motif is poorly conserved indicating that it may be involved in specific target recognition whilst the second HTH has a high degree of conservation indicating that the proteins may share a common function, such as the facilitation of RNA polymerase recruitment (Ishihama, 1993). It is thought that the members of the AraC/XylS family function by binding to the promoter regions of target genes and interacting with RNA polymerase in order initiate transcription (Ishihama, 1993). All characterised members have been identified as either positive regulators or bifunctional regulators with the exception of one protein, CelD in *E. coli*, which acts as a negative regulator of the *celABCF* operon involved in the degradation of cellobiose (Gallegos *et al*, 1997). Only one member has been shown to function by binding to an effector molecule rather than binding DNA directly, UreR (*Salmonella*

spp.) which requires the binding of urea in order to become activated (Gallegos *et al*, 1997). Members of the AraC/XylS family can be regulated themselves via two main mechanisms; they can contain a signal receptor directly upstream of the regulator which activates transcription in response to certain environmental factors or they can be regulated by another global regulator gene (Ishihama, 1993). Members of this family have been shown to be involved in a variety of diverse regulatory functions such as L-arabinose catabolism (eg. AraC, *E. coli*) and the regulation of virulence genes (eg. VirF, *Shigella dysenteriae*) (Gallegos *et al*, 1997).

1.9 AraC/XylS family transcriptional activators and antibiotic resistance in Enterobacteriaceae

1.9.1 MarA

MarA is an AraC/XylS family protein that exists within the *mar* locus in *E. coli* and is also found within other *Enterobacteriaceae* members such as *Salmonella* spp., *Enterobacter* spp., and *K. pneumoniae.* The over-expression of *marA* has been associated with the over-expression of the *acrAB* efflux pump in *E. coli* and subsequently the conferring of an MDR phenotype (Cohen *et al*, 1993; Alekshun and Levy, 1999). The *mar* locus consists of four genes and an operator-promoter region, known as *marO* (Goldman *et al*, 1996). Figure 1.3 illustrates the genomic layout, orientation and sizes of the proteins encoded by the *mar* locus in *E. coli*.

Figure 1.3. **Genetic organisation of the *mar* locus.** The genes within the *mar* locus and their corresponding protein sizes as found in *E. coli*.

marA – over-expression of *marA* alone has been shown to be capable of conferring an MDR phenotype (Goldman *et al*, 1996).

marB – over-expression of *marB* alone has no effect on antimicrobial susceptibility levels, however when over-expressed in conjunction with *marA* it appears to reduce susceptibility levels in comparison to *marA* over-expression alone (Goldman *et al*, 1996).

marC – this protein is of unknown function and its contribution to function of *marAB* is yet to be established (Goldman *et al*, 1996).

marR – the first member of the MarR family of regulators which typically act as negative regulators (Goldman *et al*, 1996). MarR functions by binding to sites within *marO*, preventing the initiation of transcription by RNA polymerase (Alekshun and Levy, 1999). The binding of MarR – *marO* can be prevented or hindered by mutations within *marR* and the presence of chemicals which interfere with the interaction, both of which result in the upregulation of *marA* (Alekshun and Levy, 1999).

X-ray diffraction analyses have shown that MarA contains two HTH motifs and binds as a monomer (Tobes and Ramos, 2002). Studies have shown that MarA binds

asymmetrically at a degenerate 20-bp DNA sequence deemed the 'marbox' (Martin *et al*, 1999). The 'marbox' has been found located both upstream of the -35 hexamer, in which case α-subunit carboxy-terminal domain (α-CTD) is required for activation, and also overlapping the -35 hexamer, in which case α-CTD activation is not required (Martin *et al*, 1999). The *marA* promoter region *marO* has also been shown to contain a 'marbox' indicating that MarA may have a degree of self regulation (Martin *et al*, 1999). Microarray analyses have identified that MarA, SoxS and Rob have overlapping regulons, regulating many identical genes such as *acrAB* and *sodA* (Martin and Rosner, 2002).

1.9.2 SoxS

SoxS is an AraC/XylS family protein found in *E. coli* and is also found within other *Enterobacteriaceae* members such as *Salmonella* spp., *Enterobacter* spp., and *K. pneumoniae*. *soxS* is expressed in response to superoxides and has been shown to upregulate the expression of the AcrAB efflux pump, similarly to MarA (Zheng *et al*, 2009). The use of redox-cycling agents, such as paraquat, have been shown *in vitro* to induce *soxS* expression (Liochev *et al*, 1999). SoxR is a MerR family protein which acts as a positive regulator of *soxS* (Amabile and Demple, 1991) and is located immediately upstream of the *soxS* gene. SoxR acts as an O^- sensor which activates *soxS* transcription upon its own oxidation (Liochev *et al*, 1999). Figure 1.4 illustrates the genomic layout, orientation and sizes of *soxS* and *soxR*.

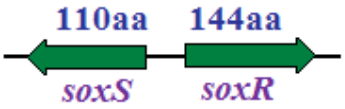

Figure 1.4. **Genetic organisation of the *sox* locus.** The genes from within the *sox* locus, *soxS* and *soxR*, and their corresponding protein sizes as found in *E. coli*.

Both global regulators, MarA and SoxS, have been shown to regulate a similar subset of genes which includes *acrAB* (Martin and Rosner, 2002). Both genes have also been shown to diminish the influence of *ompF* expression upon their own expression resulting in similar MDR phenotypic observations, likely as a result of the small RNA, *micF*, expression and additionally, both genes are understood to be activated when the bacterium is subjected to particular stressors (Demple, 1991).

1.9.3 Rob

Rob is a protein, originally discovered in *E. coli*, that binds to the right arm of the origin of replication, *oriC*. Rob is a 289 amino acid protein containing a 100 residue domain at its N-terminus that is characteristic of that found in AraC/XylS family proteins and homlogous to the domains found in *marA* and *soxS*. However the ~175 amino acid C-terminus of Rob bears no relation to any other AraC/XylS family proteins. Rob is found to be constitutively expressed; however it has shown that further over-expression (*in vitro*) results in the conferring of an MDR phenotype similar to that of *marA* or *soxS* over-expression (Bennik *et al*, 2000). Rob has been shown to contribute to the expression of *marA*, suggesting that it may play a role in

the regulation of other AraC/XylS family proteins and subsequently in mediating drug resistance via the expression of genes within the *mar* regulon (Bennik *et al*, 2000). Unlike *marA* and *soxS*, *rob* does not appear to have a local regulator up or downstream of its coding sequence and the exact role of Rob in its binding to *oriC* is unknown.

1.9.4 A shared regulon

All three of the AraC/XylS family transcriptional activators, MarA, Rob and SoxS, have been shown, by microarray analysis, to have overlapping regulons indicating a degree of redundancy exists (Martin and Rosner, 2002). All three are thought to bind the 'marbox' sequence in order to exert their regulatory effects, which in turn results in a shared regulon, to some extent (Martin and Rosner, 2002) However their regulatory effects have been shown to differ between target genes and the regulators themselves have been shown to retain some unique target genes (Amabile-Cuevas and Demple, 1991; Bennik *et al*, 2000; Martin and Rosner, 2002). This suggests that each protein is activated by a different environmental signal under conditions that are somewhat unique to each stimulus.

1.10 Transcriptional regulator RamA

1.10.1 The *ram* locus

The *ram* locus is a group of genes found in a subgroup of Enterobacteriaceae that has been associated with multidrug resistance in *K. pneumoniae* (George *et al*, 1995). Figure 1.5 illustrates the genomic layout, orientation and sizes of genes from within the *ram* locus in *K. penumoniae*.

Figure 1.5. **Genetic organisation of the *ram* locus in *K. pneumoniae* and *Enterobacter* spp.** The genes contained within the *ram* locus and their corresponding protein sizes.

It is important to note that not all bacterial species containing the *ram* locus share the same genomic layout as *K. pneumoniae* and *Enterobacter* spp. *Salmonella* spp. lack the *romA* gene (Figure 1.6).

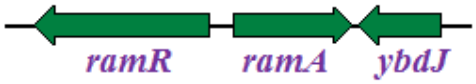

Figure 1.6. **Genetic organisation of the *ram* locus in *Salmonella* spp.** The *ram* locus as found in *Salmonella* spp.

1.10.1.1 RamA

RamA is an AraC/XylS family transcriptional activator found in *Enterobacter* spp., *Salmonella* spp. and *K. pneumoniae*. It shares a distinct homology with the *E. coli* transcriptional activators MarA and SoxS (Figure 1.7). Although *marA* and *soxS* are also present in *Enterobacter* spp., *Salmonella* spp. and *K. pneumoniae*, *ramA* is notably absent from *E. coli*.

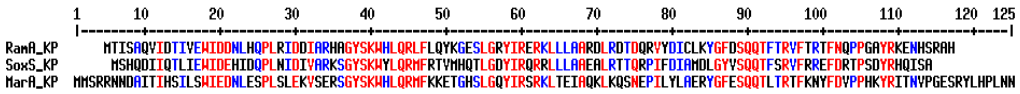

Figure 1.7. **Protein alignment of MarA, RamA and SoxS from *K. pneumoniae*.** A Multalin alignment of RamA, MarA and SoxS protein sequences obtained from the *K. pneumoniae* MGH 78578 genome sequence. Highlighted in red are the conserved regions that characterise the proteins as members of the AraC/XylS family.

RamA was originally discovered in *K. pneumoniae* by George *et al* (1995) where it was shown that when the *ramA* gene was transformed into to a susceptible *E. coli* strain, it conferred an MDR phenotype. George *et al* (1995) also selected for *ramA* mutants (over-expressers) using chloramphenicol but found that the sequence of the mutant *ramA* was identical to that of the wild-type strain, indicating that the causative mutation was not within the gene itself. A subsequent study by Schneiders *et al* (2003) found that *ramA* over-expression may be positively linked to *acrA* over-

expression. Further studies in *K. pneumoniae* by Ruzin *et al* (2005) supported this hypothesis by observing that *ramA* over-expression correlated with *acrAB* over-expression, leading to the premise that *acrAB* may be under the transcriptional control of *ramA*.

1.10.1.2 RomA

RomA is a putative outer membrane protein encoded downstream of *ramA* in *K. pneumoniae* and *Enterobacter* spp. but is notably absent from the *ramA* locus in *Salmonella* spp. (Komatsu *et al*, 1990). Komatsu *et al* (1990) hypothesised that *romA* encodes an outer membrane protein and was responsible for conferring an MDR phenotype. However the exact function of RomA remains elusive as it bears no strong homology to any other known outer membrane proteins and when its putative protein sequence is run through NCBI's blast software, RomA only bears vague similarity to a metallo-β-lactamase. Currently there is no solid evidence that supports either hypothesis for the role of RomA and its association, if any, with RamA remains unknown.

1.10.1.3 RamR

RamR is a TetR family protein, which are a family of proteins that typically act as transcriptional repressors. *ramR* is located immediately downstream of *romA*, encoded on the opposite DNA strand. *ramR* was hypothesised to encode a repressor of *ramA* in a study by Abouzeed *et al* (2008). In the this study it was shown that the

deletion of *ramR* in *Salmonella* led to an increase in MICs to a broad range of antibiotics and the upregulation of *ramA* expression, as measured by RT-PCR. The complementation of *ramR* restored the activities of the antibiotics and so RamR was understood to be acting as a local repressor of *ramA* (Abouzeed *et al*, 2008).

1.10.1.4 YbdJ

ybdJ is located immediately downstream from *ramA* and encodes a putative protein. The function of YbdJ is unknown, it is not known whether it plays a role in the regulation of *ramA* or is even included within its regulon.

1.10.2 Which regulator?

All four of the previously mentioned AraC/XylS regulators, MarA, SoxS, RamA and Rob, are capable of cross regulation of their gene sets and conferring an MDR phenotype (Martin and Rosner, 2002; Schneiders *et al*, 2003). However this does not mean that all four are expressed simultaneously within a cell or by a particular species of bacterium. The over-expression of these genes *in vivo* appears to be somewhat species specific; for example *rob* is constitutively expressed in the bacteria listed in Table 1.6 and, *in vitro,* further over-expression has been shown to confer an MDR phenotype (Bennik *et al*, 2000). However to date there have been no reports of *rob* over-expressers found in nature. Similarly *marA* or *soxS* over-expressers appear rarely in *Salmonella* spp. and have not been reported in *K. pneumoniae,* unless

induced *in vitro*. This suggests that *Salmonella* spp. and *K. pneumoniae* may favour the over-expression of *ramA* to regulate the shared regulons.

	MarA			SoxS			RamA			Rob		
	Present	**Over-expression**	**Reference**	**Present**	**Over-expression**	**Reference**	**Present**	**Over-expression**	**Reference**	**Present**	**Over-expression**	**Reference**
E. coli	+	+	Cohen *et al*, 1993.	+	+	Amabile-Cuevas and Demple, 1991.	-	N/A	N/A	+	-[a]	N/A
***Enterobacter* spp.**	+	+	Chollet *et al*, 2002.	+	+	Masi *et al*, 2006.	+	+	Chollet *et al*, 2004.	+	-[a]	N/A
K. pneumoniae	+	-[a]	N/A	+	-[a]	N/A	+	+	George *et al*, 1995.	+	-[a]	N/A
***Salmonella* spp.**	+	+	Eaves *et al*, 2004.	+	+	Koutsolioutsou *et al*, 2001.	+	+	van der Straaten *et al*, 2004.	+	-[a]	N/A

Table 1.6. **AraC/XylS regulators in Enterobacteriaceae.** Shows the presence of the AraC/XylS regulators in *E. coli*, *Enterobacter* spp., *K. pneumoniae* and *Salmonella* spp. and whether over-expressers have been reported. + = present/over-expressed, - = absent/not over-expressed, -[a] = over-expression in clinical isolates has not been reported previously.

1.11 Hospital acquired infections (HAIs)

1.11.1 Emergence of HAIs

A hospital acquired infection (HAI), also known as healthcare associated infection or nosocomial infection, is defined by the EU as "any disease or pathology related to the presence of an infectious agent or its products as a result of exposure to healthcare facilities or healthcare procedures". The majority of HAIs are caused by opportunistic pathogens that pose no risk to healthy individuals. Hospitalised patients are of particularly high risk of contracting infections due to a high prevalence of pathogens, high prevalence of immunocompromised patients and the presence of efficient mechanisms of transmission. Modern medicine and medical advances has meant that people are now living longer and surviving with medical conditions that would previously have been fatal and so increasing the population vulnerable to HAIs. Patients within intensive care units (ICU) are at the greatest risk of acquiring HAIs due to both their immune compromised state and their being subjected to invasive medical procedures (Spencer, 1994). Despite the highly publicised emergence of the Gram-positive 'superbugs', primarily methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals, Gram-negative pathogens have continued to steadily increase in their incidence (Boucher *et al*, 2009). Several Gram-negative species that were previously thought to have been of little clinical significance are now the cause of great concern due to their rapid development of antibiotic resistance, prompting a marked escalation into the research of their resistance mechanisms (Boucher *et al*, 2009).

1.11.2 Incidence and costs of HAIs

The reported incidences of HAIs differs between studies and geographical regions but have been estimated by the Center For Disease Control (CDC) to encompass 5% to 10% of hospitalised patients (Emori and Gaynes, 1993). In England around 9% of patients are estimated to have an HAI at any one time, resulting in 300,000 infections and directly causing at least 5000 deaths per year (National Audit Office, 2004). These figures can be considered to be an underestimation of the true extent of HAIs as, in addition, an unknown number of discharged patients have an infection related to their hospital stay. HAIs are estimated to result in a 2.5 times longer hospital stay with an attributable mortality of 1-2.7% (Emori and Gaynes, 1993). The global incidence of HAIs appears to be on the increase; for example, in the US the rates of nosocomial infections, taking into account shorter hospital stays, has increased 36% over the past 20 years (Weinstein, 1998). Such a high incidence of HAIs inevitably results in high costs including: direct costs, such as medication, diagnostic testing and staff costs; indirect costs, such as patient loss of income; and personal costs such as the physical and emotional pain and suffering of the patient. This is estimated to cost the National Health Service (NHS) in excess of £1bn per year in addition to the costs to the patient (National Audit Office, 2004). A study by Daxboeck *et al* (2006) performed in Vienna General Hospital, Austria, compared the attributable costs of infections caused by MDR Gram-negative infections against MRSA infections. They reported that the median costs of a MDR Gram-negative infection to be £18115 with an average hospital stay of 42 days compared to £6624 and 37 days for MRSA infections (Daxboeck *et al*, 2006).

1.11.3 Transmission

HAIs can be transmitted via a number of different routes including through medical devices and equipment (eg catheters), and between patients and hospital workers. Disease causing pathogens can become incorporated into the normal flora of hospital, thus aiding their transmission. In recent years attempts have been made by numerous healthcare authorities to break the transmission and subsequently, the incidence of HAIs via campaigns to increase the awareness of both healthcare workers and the general public, to the importance of handwashing. One study reported that alcohol rubs resulted in lower bacterial counts when compared to traditional handwashing with soap (Girou *et al*, 2002) and subsequently the use of alcohol–based rubs have now been introduced to most UK hospitals.

1.11.4 Types of HAI

The four major types of HAI are as follows: urinary tract infection (UTI), surgical site infection (SSI), respiratory tract infection (RTI) and bloodstream infections (BSI). In the USA the CDC estimate that the total HAIs are comprised as follows (Klevens *et al*, 2007):

UTI – 32%

SSI – 22%

RTI – 15%

BSI – 14%

Others (eg skin infections) – 17%

UTIs are the most common type of HAI largely due to a combination of the urinary catheterisation of patients and the presence of multiple organism types in the hospital environment. Around 80% of UTIs are caused by *E. coli*, but *P. aeruginosa* and *K. pneumoniae* are also common causes (Nicolle, 2008).

A study by Gaynes *et al* (2005) examined the incidences of bacterial infections from ICUs in the US over a 28 year period from 1975 to 2003. Gram-negative pathogens were found to predominate total nosocomial infections comprising 53.4-79.5% of each type of infection in 1975 and 23.8-72.9% in 2003 (Gaynes *et al*, 2005). As shown in Table 1.7, the incidence of Gram-negative infections appears to have dropped slightly for UTI, SSI and RTI over the study period, although Gram-negative pathogens remained dominant. However the incidence of Gram-negative BSIs have more than halved over the 28 year study period. This phenomenon could be attributed to the emergence of MRSA as a nosocomial pathogen (Gaynes *et al*, 2005). *K. pneumoniae* infection rates have remained steady for each site of infection with the exception of UTIs which have more than doubled in their incidence.

		1975	2003
Gram-negative Pathogens	UTI	78.5	72.9
	SSI	53.4	41.9
	RTI	79.5	66.0
	BSI	57.1	23.8
K. pneumoniae	UTI	4.6	9.8
	SSI	2.7	3.0
	RTI	6.4	7.2
	BSI	4.5	4.2

Table 1.7. **Comparison of Gram-negative and *K. pneumoniae* infection rates in ICUs between 1975 and 2003.** Figures relate to the percentage of isolates recovered from each type of infection (adapted from Gaynes *et al*, 2005).

1.12 Antibiotic resistant HAIs

1.12.1 Antibiotic resistant infections

Acquired antibiotic resistance can be considered one of the greatest problems in hospitals today. It is estimated that there are over 2m nosocomial infections in the US each year and 50-60% of these are caused by resistant strains (Jones, 2001).

1.12.2 The main culprits

Six pathogens have been reported to cause the majority of antibiotic resistant hospital infections in the United States; *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A baumannii*, *P. aeruginosa* and *Enterobacter* spp. (Boucher *et al*, 2009). Antibiotic

resistant infections result in increased mortality rates and longer hospitals stays with respect to infections caused by their susceptible counterparts (Maudlin *et al*, 2010).

1.12.3 Combating resistance

Increasing reports of "hospital superbugs" in the media have led many scientists and clinicians to believe that modern medicine is losing the battle against antibiotic resistant bacteria, and current reports suggest that this view does have some basis (Levy and Marshall, 2004; Falagas and Bliziotis, 2007). A study by Safdar *et al* (2002) reported a 25% rate of ESBL carrying Enterobacteriaceae for ICU patients. The use of antibiotics has undoubtedly caused many bacterial species to undergo strong evolutionary pressure and has likely to have sped up such evolutionary processes involved in the development of resistance mechanisms. Studies have shown that the development of antibiotic resistance can be directly correlated with the introduction of antibiotics into medical usage and it has been shown that when antibiotic usage is restricted, the incidences of resistance are reduced (Struelens *et al*, 1999; De Man *et al*, 2000). The current limited state of antibiotic therapy indicates that new therapies and new drug targets are a necessity in order to ensure that many medical treatments and surgeries remain a viable option in the present as well as the future. In the quest for new therapies it has become essential that we understand the molecular basis for antibiotic resistance in an effort to both overcome this phenotype and limit its spread.

1.12.4 Interventions

In the absence of new antimicrobials, UK medical trusts have employed a number of measures with which they aim to exert some control over the current issues of HAIs and antibiotic resistance.

-Infection control measures

Several strategies have been put in place amongst healthcare staff with the aim of preventing the spread of infections in the UK. Most of these strategies are centred around improving cleanliness and hygiene within the medical settings. The combined use of alcohol-based hand gels and improved infection control education amongst are expected to limit the spread of nosocomial infections (NHS, 2003).

-Resistance development control measures

The large scale spread of resistance to antibiotics has been attributed, at least in part, to the inappropriate prescribing and administration of antibiotics. To combat this problem the Health Protection Agency (HPA) have introduced antibiotic prescription guidelines alongside continuous monitoring of infections and resistance in order to detect the early development of any patterns so that appropriate interventions can be put in place to limit their spread (HPA, 2010).

The employment of these strategies can help to reduce or limit infection and resistance rates in UK hospitals, however on their own they are inadequate measures for combating the ongoing evolution of bacteria.

1.13 *Klebsiella* spp.

1.13.1 The *Klebsiella* genus

The Danish scientist Hans Christian Gram (1853-1938) developed a technique in 1884 which could distinguish between two major types of bacteria which shared similar clinical symptoms; *Streptococcus pneumoniae* and *K. pneumoniae*. This technique was thereafter known as the Gram stain and is still employed today as a basic means to distinguish between Gram-positive and Gram-negative bacteria. The *Klebsiella* genus itself was named after the German bacteriologist/pathologist Edwin Klebs (1834-1913) whose earlier work with fellow German bacteriologist Friedrich Loeffler (1852-1915) resulted in the identification of *Corynebacterium diphtheriae*, also known as Klebs-Loffler bacillus, as the cause of diphtheria. Trevisan (1885) named the genus *Klebsiella* after him in honour of his work.

Members of the genus *Klebsiella* spp. are Gram-negative rods belonging to the Enterobacteriaceae family. The common characteristics of *Klebsiella* spp. include that they are non-motile, oxidase negative and encapsulated. The polysaccharide capsule shared by the *Klebsiella* genus is an important pathogenicity and virulence determinant, providing the bacterium with protection from phagocytosis and against

the host's antibodies. *Klebsiella* spp. are ubiquitous in nature and commonly found in water, soil and the mucosal membranes of both animals and humans (Gupta *et al*, 2003). There are seven recognised species within the *Klebsiella* genus according to Orskov's classification; *K. planticola*, *K. pneumoniae*, *K. ornithinolytica*, *K. oxytoca*, *K. ozaenae*, *K. rhinoscleromatis* and *K. terrigena* although it should be noted that *K. ozaenae* and *K. rhinoscleromatis* are considered to be subspecies of *K. pneumoniae* by many scientists.

Klebsiella spp. are considered to be opportunistic pathogens primarily causing infections in those who are immune compromised and those with underlying health conditions such as diabetes, lung disease and alcoholics (Anonymous, CDC, 2004). *Klebsiella* spp. are reported to be responsible for 7-10% of bloodstream infections in Europe, North America and South America according to data collected by the SENTRY Antimicrobial Surveillance Program (Alves *et al*, 2006). The vast majority of human infections are caused by *K. pneumoniae* with *K. oxytoca* and *K. rhinoscleromaitis* causing a minority of infections by comparison (Podschun and Ullmann, 1998). The other members of the *Klebsiella* genus are not thought to cause infections in humans with the exception of *K. ozaenae* which is believed to cause disease of the nasal passages, although has not yet been confirmed as a causative agent (Anonymous, CDC, 2004).

1.13.2 Virulence

Klebsiella spp. possess a number of properties that enhance their virulence and pathogenicity.

1.13.2.1 Polysaccharide capsule

The bacterial capsular polysaccharrides are perhaps the most important virulence factor for *Klebsiella* spp. The polysaccharide capsule consists of repeating subunits of sugars and uronic acids. Capsular polysaccharide (CPS) and lipopolysaccharides (LPS) are the two most important components of the polysaccharide capsule. *Klebsiella* spp. express two cell surface antigens; O, a component of LPS of which there are 9 varieties, and K, a CPS of which there are over 80 varieties. The polysaccharide capsule acts to protect the bacterium from both polymorphonuclear granulocyte mediated phagocytosis and bactericidal serum factors (Podschun and Ullmann, 1998).

-CPS

There are currently over 80 different serotypes of *Klebsiella* spp., although some have been shown to more virulent than others (Gupta *et al*, 2003). K antigens lacking repetitive mannose and rhamnose structures have been found to exhibit increased virulence due to their not being recognised by the surface lectin of macrophages (Podschun and Ullmann, 1998). The K2 antigen lacks the repetitive

mannose and rhamnose structures, perhaps the reason why K2 is the predominant serotype worldwide (Podschun and Ullmann, 1998).

-LPS

LPS is found in the outer membrane of all Gram-negative bacteria and comprises three components; the O antigen, the core oligosaccharide, and lipd A. LPS itself is an endotoxin and the lipid A component has toxic properties, eliciting a potent immune response often causing fever in the infected host. LPS activates the complement system by one of two pathways; the classical pathway via the lipid A component or the alternative pathway via the O antigen component.

1.13.2.2 Fimbriae

The ability of a bacterium to form biofilms on surfaces and medical devices is considered a major factor in the spread of nosocomial infections. Adhesion is an essential first step for biofilm production and is mediated by fimbrial adhesins in *Klebsiella* spp. Fimbriae allow the bacterium to adhere to the surface to cells, thus aiding their ability to cause disease. *Klebsiella* spp. typically express two types of fimbriae; type 1 is the most common, found in most members of the Enterobacteriaceae, and is typically found in most clinical strains, type 3 is less common and was typically thought to only be present in plant strains, although have also been found in strains causing human infection (Podschun and Ullmann, 1998). Type 1 fimbriae have been shown to mediate adhesion to mannose-containing

structures. It has been shown that type 1 fimbriae are essential virulence factors in causing UTIs in animal models but appear to play no role in mediating biofilm production, in contrast to that found in *E. coli* (Pratt and Kolter, 1998; Schroll *et al*, 2010). Type 3 fimbriae have been shown to have no influence on pathogencity in an animal UTI model but have been shown to be important for mediating biofilm production in *in vitro* studies (Schroll *et al*, 2010).

1.13.2.3 Siderophores

Siderophores are iron chelating compounds which are secreted by micro-organisms as a means of binding and acquiring iron, an essential element for bacterial growth. *Klebsiella* spp. produce two types of siderophores; enterobactin and aerobactin, both of which have been identified as important virulence factors (Podschun and Ullmann, 1998).

1.14 *K. pneumoniae*

1.14.1 Clinical significance

K. pneumoniae can be differentiated from other *Klebsiella* spp. by the fact that it can ferment lactose and is indole negative. Identification can additionally be based upon the nucleotide sequences of *gyrA*, *parC* and *rpoB* genes, after which *K. pneumoniae* strains fall into one of four phylogenetic groups; KpI, KpII-A, KpII-B, and KpIII (Alves *et al*, 2006). *K. pneumoniae* can cause a variety of infections in humans

including; pneumonia, bacteriaemia, UTI, wound infections and GI infections. Although *K. pneumoniae* has been associated with community-acquired infections such as pneumonia, the majority of infections caused by this bacterium are associated with hospitalisation (Podshun and Ullmann, 1998). *K. pneumoniae* is an important cause of HAIs, especially in the neonatal ICU where mortality rates can reach up to 70% (Gupta *et al*, 2003). A study by Jones *et al* (2004) examined the incidence of bacterial infections in ICU in five countries; the USA, Canada, Italy, Germany and France. This study found that the incidence rates of *K. pneumoniae* infections differed between countries, ranging from 3.5-5.8%, but was the third most commonly isolated Gram-negative organism behind *P. aeruginosa* and *E. coli* across all five countries (Jones *et al*, 2004). The incidence of *K. pneumoniae* infections has increased significantly over the past 20 years, coinciding with the spread of ESBLs, which are increasingly harboured in clinical strains (Gupta *et al*, 2003). A study by the National Healthcare Safety Network in the USA over a 12 month period from 2006-2007 revealed that *K. pneumoniae* comprised 5.8% of HAIs and 7.7% of catheter-associated UTIs (Hidron *et al*, 2008). The same study showed that 21.2% of those *K. pneumoniae* isolates were resistant to cephalosporins and 10.1% exhibited resistance to carbapenems (Hidron *et al*, 2008). This identifies that antibiotic resistance is a major factor in the success of *K. pneumoniae* as pathogens.

K. pneumoniae has been identified as a primary cause of liver abscesses in Asia (Yu *et al*, 2008). A study by Yu *et al* (2008) showed that 72% of strains isolated from *K. pneumoniae* caused liver abscesses were of capsular seroptype K1 or K2. The acquisition of *K. pneumoniae* caused liver abscess has been associated with

underlying medical conditions, primarily diabetes mellitus, although the reasons for this association have not yet been identified (Lin *et al*, 2004).

1.14.2 *K. pneumoniae* risk factors

As with any bacterium capable of causing nosocomial infections, a number of risk factors are associated; in *K. pneumoniae* this is primarily the existence of an immune compromised host.. There are a number of risk factors that have been identified to be associated with the acquisition of ESBL-producing *K. pneumoniae*: invasive procedures including abdominal surgery, arterial and central venous catheterisation, urinary catheterisation, and mechanical ventilation; low birth weight in infants; prolonged hospital stays; prior antibiotic use, particularly cephalosporins and aminoglycosides; and colonisation of the GI tract (Jacoby, 1998; Lautenbach *et al*, 2001).

1.15 Aims of this thesis

- To examine the expression of the transcriptional activator RamA and its contribution to antibiotic resistance in *K. pneumoniae*.

- To decipher the role of RamR in the regulation of *ramA* and *acrA* expression.

- To examine the effect of antibiotics and other compounds on both *ramA* and *ramR* and their effects in conferring a multidrug resistant phenotype.

- To investigate the mechanisms and development of antibiotic resistance, specifically to the carbapenem antibiotics, in two specific *K. pneumoniae* strains.

Chapter 2: Materials and Methods

2.1 Bacterial strains

2.1.1 All bacterial strains used in this study

K. pneumoniae strains investigated in this study were of laboratory, clinical and environmental origins, and are listed in Table 2.1. Standard β-lactamase producing strains are listed in Table 2.2 and other standard strains used in this study are listed in Table 2.3. All strains had been previously speciated by other laboratories.

***K. pneumoniae* Strains**	**Laboratory**	**Clinical**	**Environmental**	**Reference**
K1		+		This study.
K2		+		This study.
Ecl8	+			George *et al*, 1995.
Ecl8 Mdr1	+			George *et al*, 1995.
S7		+		Schneiders *et al*, 2003.
S8		+		Schneiders *et al*, 2003.
CG43		+		Lai *et al*, 2000.
MGH 78578		+		Ogawa *et al*, 2005.
Kp342			+	Fouts *et al*, 2008.
S28		+		Schneiders *et al*, 2003.
S29		+		Schneiders *et al*, 2003.

Table 2.1. ***K. pneumoniae* strains investigated in this study.**

Strain	**pI**	**Source**
E. coli TEM-1	5.4	SGBA[1]
E. coli TEM-2	5.6	SGBA[1]
E. coli TEM-3	6.3	SGBA[1]
E. coli SHV-1	7.6	SGBA[1]
E. coli SHV-3	7.0	SGBA[1]
E. coli SHV-5	8.2	SGBA[1]

Table 2.2. **Standard β-lactamase producing strains.**

[1]SGBA = Sebastian Amyes

Strain	Marker	Plasmid Size	Source
E. coli DH5α			SGBA[1]
E .coli K12 MG1655			SGBA[1]
E. coli ATCC 25922			SGBA[1]
E. coli K12 J62-2	Rif^R		SGBA[1]
E. coli DH5α/pACYC177	Amp^R, Kan^R	3,941bp	SGBA[1]
K. pneumoniae CTX-M-15 1			SGBA[1]
K. pneumoniae CTX-M-15 2			SGBA[1]
K. pneumoniae ATCC 13883			Pro-Lab Diagnostics
K. pneumoniae KPC-3			AH[2]

Table 2.3. **Standard bacterial strains.**

[1]SGBA = Sebastian Amyes; [2]AH = Ahmed Hamouda

2.1.2 Storage of strains

Bacterial strains were inoculated onto MacConkey agar plates and incubated overnight at 37ºC. A single colony was inoculated into vials containing 5ml of LB broth and incubated overnight. 900µl of strain culture was placed into a Cryovial with 100µl of sterile 50% glycerol to give a final volume of 5%. The Cryovials were mixed by vortexing and stored at -80ºC.

2.2 Media, buffers and reagents

2.2.1 Media and buffers

All growth media and buffers used were sterilised by autoclaving at 15 lbs psi at 121°C for 15 mins.

2.2.2 Solid media

LB agar, IsoSensitest (IST) agar, nutrient agar and MacConkey agar were obtained from Oxoid (Hampshire, UK) and prepared according to the manufacturer's instructions. Agar was cooled to 50ºC before being poured into sterile petri dishes (Sterilin Ltd, Staffordshire, UK) and allowed to set. Plates were either used immediately or stored at 4ºC.

2.2.3 Liquid media

LB broth and nutrient broth were obtained from Oxoid (Hampshire, UK) and prepared according to the manufacturer's instructions.

2.2.4 Reagents

All reagents were obtained from either Sigma Chemicals (Dorset, UK) or Fisher Scientific (Leicestershire, UK) unless stated otherwise.

2.3 Antimicrobial agents

2.3.1 Antibiotics

The antimicrobial agents used in this study were obtained from suppliers as listed in Table 2.4 and dissolved or diluted in an appropriate solvent according to the British

Society for Antimicrobial Chemotherapy susceptibility testing guidelines (Andrews, 2001).

Antimicrobial Agent	Supplier
Amikacin	Sigma-Aldrich (Dorset,UK)
Ampicillin	Sigma-Aldrich (Dorset,UK)
Aztreonam	Sigma-Aldrich (Dorset,UK)
Cefoxitin	Sigma-Aldrich (Dorset,UK)
Ceftazidime	Sigma-Aldrich (Dorset,UK)
Chloramphenicol	Sigma-Aldrich (Dorset,UK)
Ciprofloxacin	Sigma-Aldrich (Dorset,UK)
Clavulanic acid	Sigma-Aldrich (Dorset,UK)
Colistin	Sigma-Aldrich (Dorset,UK)
Ertapenem	Merck (Nottingham, UK)
Gentamicin	Sigma-Aldrich (Dorset,UK)
Imipenem	Merck (Nottingham, UK)
Kanamycin	Sigma-Aldrich (Dorset,UK)
Meropenem	Astra Zeneca (London, UK)
Norfloxacin	Sigma-Aldrich (Dorset,UK)
Piperacillin	Sigma-Aldrich (Dorset,UK)
Rifampicin	Sigma-Aldrich (Dorset,UK)
Sulbactam	Sigma-Aldrich (Dorset,UK)
Tetracycline	Sigma-Aldrich (Dorset,UK)
Tigecycline	Wyeth (Maidenhead, UK)
Tobramycin	Faulding (Warwickshire, UK)

Table 2.4. **Antimicrobial agents used in this study.**

2.3.2 Antibiotic discs

All antibiotic discs were obtained from Mast Laboratories Ltd, Liverpool, UK.

2.4 Antibiotic susceptibility testing

2.4.1 Minimum inhibitory concentrations (MICs)

MIC testing was carried out using IST agar according to BSAC guidelines (Andrews, 2001). Bacterial strains were grown overnight at 37°C in LB broth and diluted into 0.9% NaCl to approximately 10^7 cfu/ml. A Denley multipoint inoculator (Denley, Surrey, UK) was used to inoculate a 1µl volume onto the surface of the agar plates to give a final inoculum of approximately 10^4 cfu/spot. A plate containing no antibiotic was used as a positive control. The plates were then incubated in air at 37°C for 18 hours. The MIC was deemed to be the lowest antibiotic concentration to inhibit all visible growth. *E. coli* ATCC 25922 was used as a control.

2.4.2 Disc sensitivity testing

Disc sensitivity testing was performed on IST agar plates and interpreted as sensitive, intermediate or resistant according to the BSAC disc sensitivity testing guidelines (Andrews, 2007).

2.4.3 Chlorpromazine synergy determination

To determine the presence or absence of synergy between antimicrobials and chlorpromazine (CPZ) (Sigma-Aldrich, Dorset, UK), the fractional inhibitory concentration (FIC) index was used as a guide.

FIC values were calculated using the following formula and interpreted according to Table 2.5 (Gould *et al*, 1991).

$$\text{FIC} = \frac{\text{MIC of Agent A (combination)}}{\text{MIC of Agent A (single)}} + \frac{\text{MIC of Agent B (combination)}}{\text{MIC of Agent B (single)}}$$

FIC Value	Interpretation
≤0.5	Synergy
>0.5 - 1.0	Addition
>1.0 - ≤4.0	Indifference
>4.0	Antagonism

Table 2.5. **FIC interpretation.**

2.5 Polymerase chain reactions (PCRs)

2.5.1 Primer design

Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3) or otherwise were taken from previously published work. Primers were synthesised by Eurofins MWG Operon (London, UK). Table 2.6 lists all of the primers used in this study.

Primers	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Reference
Gene Detection and Sequencing			
AmpC_F & AmpC_R	ATCAAAACTGGCAGCCG	GAGCCCGTTTTATGCACCCA	Kaczmarek *et al*, 2006.
CTX_MA & CTX_MB	CGCTTTGCGATGTGCAG	ACCGCGATATCGTTGGT	Dutour *et al*, 2002.
GES_F & GES_R	ATGCGCTTCATTGACGCAC	CTATTTGTCCGTGCTCAGG	Park *et al*, 2006.
GIM_F & GIM_R	TCGACACACCTTGGTCTGAA	AACTTCCAACTTTGCCATGC	Ellington *et al*, 2007.
IMP_F & IMP_R	GGAATAGAGTGGCTTAAYTCTC	CCAAACYACTASGTTATCT	Ellington *et al*, 2007.
KPC_F & KPC_R	CAGCTCATTCAAGGGCTTTC	GGCGGCGTTATCACTGTATT	This work.
NDM_F & NDM_R	GAAGCTGAGCACCGCATTAG	GACTTGGCCTTGCTGTCCTT	This work.
NMC/IMI_F & NMC/IMI_R	ATGTCATTAGGTGATATGGC	GCATAATCATTTGCCGTACC	Kaczmarek *et al*, 2006.
OXA_F & OXA_R	TATCTACAGCAGCGCCAGTG	CGCATCAAATGCCATAAGTG	This work.
PER_F & PER_R	ATGAATGTCATTATAAAAGC	AATTTGGGCTTAGGGCAGAA	Park *et al*, 2005.
SHV_F & SHV_R	CTTTCCCATGATGAGCACCT	CGCTGTTATCGCTCATGGTA	This work.
SIM_F & SIM_R	TACAAGGGATTCGGCATCG	TAATGGCCTGTTCCCATGTG	Ellington *et al*, 2007.
SME_F & SME_R	TAGAGGAAGACTTTGATGGG	GCATAATCATTCGCAGTACC	Kaczmarek *et al*, 2006.
SPM_F & SPM_R	AAAATCTGGGTACGCAAACG	ACATTATCCGCTGGAACAGG	Ellington *et al*, 2007.
TEM_F & TEM_R	CGCCGCATACACTATTCTCA	TTGCCGGGAAGCTAGAGTAA	This work.
VEB_F & VEB_R	CGACTTCCATTTCCCGATGC	GGACTCTGCAACAAATACGC	Park *et al*, 2005.
VIM_F & VIM_R	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	Ellington *et al*, 2007.
OmpK35_F & OmpK35_R	CAGACACCAAACTCTCATCAATGG	AGAATTGGTAAACGATACCCACG	Kaczmarek *et al*, 2006.
OmpK36_F & OmpK36_R	CAGCACAATGAATATAGCCGAC	GCTGTTGTCGTCCAGCAGGTTG	Kaczmarek *et al*, 2006.
RamR_F & RamR_R	CATCCCGGAGGCTTTATGAT	CGCTCGACCTTAAACACGTC	This Work.
RT-PCR Analysis			
RamA_RTF & RTR	CTGCAACGGCTGTTTTTACA	GTGGTTCTCTTTGCGGTAGG	This work.
RomA_RTF & RTR	GTTCACCGGGCAGAAAAATA	GAGCCAGACCATCACATCCT	This work.
RamRTF & RamRTR	GGCTCGTCCAAAGAGTGAAG	CGTTCCACATGGCTTCAAA	This work.
AcrA_RTF & AcrA_RTR	GTCCTCAGGTCAGTGGCATT	GGTGCCCAACAGTTTCTGAT	This work.
OmpK34_RTF & OmpK34_RTR	TAACGATCAGCTTGGTGCTG	ATCGTCAGTGATCGGGTAGC	This work.
OmpK35_RTF & OmpK35_RTR	AAAACGGCAACAAACTGGAC	AGACGGGTTTTTGTGGTCTG	This work.
OmpK36_RTF & OmpK36_RTR	GCGCTCTGTCTCCTACCAAC	GGTGTACTGAGTGGCCAGGT	This work.
YbdJ_F & R	TGAAACATCCACTGGAAACG	GCGCTAAGAACCACAGACAA	This work.
16S_RTF & 16S_RTR	CAGCCACACTGGAACTGAGA	GTTAGCCGGTGCTTCTTCTG	This work.
Cloning			
RamRF2 & RamRR2	AACTGCAGTCGTCAAGACGATTTTCAATTTT	AAAAGTACTAGTGTTTCCGGCGTCATTAG	This work.
RomAF2 & RomAR2	AACGAGTACTATGCCGGAAATTGATCTG	AACGCTGCAGCAGCAGGCGCCACGCGCG	This work.

Table 2.6. **The primers used in this study.**

2.5.2 Genomic DNA preparation for PCR

DNA was prepared for PCR by resuspension of 1-2 colonies, picked from an agar plate, in 100µl of sterile distilled water. The suspensions were then boiled in a waterbath for 10 mins before being chilled on ice. 1µl of this DNA preparation was used in each PCR reaction.

2.5.3 PCR reaction components

PCR reactions were performed in total volumes of 50µl. Table 2.7 lists the reaction components in the order they were added to a mastermix before being aliquoted into a sterile 0.5ml thin walled PCR tube containing the DNA template. A negative control was included which contained all reaction components minus any DNA. The PCR tubes were mixed by pulsing in a microcentrifuge prior to being placed in a thermal cycler. All PCRs were performed using Go-Taq polymerase (Promega, Southampton, UK).

Component	Stock Concentration	Volume Added (µl)	Final Concentration
10x Buffer	200mM Tris-HCl, 500mM KCl	5	1x buffer; 20mM Tris-HCl, 50mM KCl
$MgCl_2$	50mM	1.5	1.5mM
dntp mix	1mM	1	20pM
Forward primer	10µM	0.5	100pM
Reverse primer	10µM	0.5	100pM
Taq polymerase	5U/µl	0.25	1.25U
DNA template	N/A	1	N/A
SDW	N/A	40.25	N/A

Table 2.7. **PCR reaction components.** N/A – not applicable

2.5.4 PCR cycling parameters

PCR cycling was performed in an GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Warrington, UK). Specific cycling parameters are given in Table 2.8. The annealing temperatures and number of cycles varied depending upon the primer length, GC content and target gene.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30s	} Various
Annealing	Various	30s	
Extension	72	30s	
Final Extension	72	7 min	1
Hold Temperature	4	Infinity	

Table 2.8. **PCR cycling parameters.** The typical parameters used for PCR reactions. The annealing temperature used was dependent upon the primer sequence. N/A – not applicable

2.6 Agarose gel electrophoresis of DNA

2.6.1 PCR amplified DNA

PCR amplified DNA was electrophoresed in 1.5% w/v agarose gels made in 1X TAE buffer (40mM Tris-acetate, pH8.0; 2mM EDTA). Electrophoresis was performed in Bio-Rad Mini Sub Cell horizontal gel units in 1X TAE running buffer, at room temperature and 100V for 30 – 40 mins.

2.6.2 Plasmid DNA

Plasmids preparations were electrophoresed in 0.85% w/v agarose gels made in 0.5X TBE buffer (89mM Tris base; 89mM Boric acid; 2mM EDTA). Electrophoresis was performed in Bio-Rad Maxi Sub Cell horizontal gel units in 0.5X TBE running buffer, at 4°C and 90V for 15 hours.

2.6.3 Sample preparation

Samples were mixed with loading buffer (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0)) in a 5:1 ratio (DNA:loading buffer) prior to loading onto the gel. A 100bp or 1kb DNA marker (Promega, Southampton, UK) was run on the gels alongside the samples to facilitate size estimation.

2.6.4 Nucleic acid gel staining and photographing

Gels were stained in either ethidium bromide at a concentration of 50mg/L for half an hour or GelRed (Biotium, Hayward, CA, USA) according to the manufacturer's instructions. Gels were viewed and photographed using the Bio-Rad GelDoc system.

2.7 DNA/RNA analysis

2.7.1 Purification of PCR products

Purification of PCR products was performed using the QIAquick PCR Purification Kit (Qiagen, Sussex, UK) according to the manufacturer's instructions. The purified DNA was stored at -20°C.

2.7.2 Estimation of DNA/RNA concentration

The concentration of DNA and RNA were estimated by loading a 2µl sample onto the NanoDrop 2000 spectrophotometer (Thermo Scientific, Cramlington, UK) and taking the average of two independent readings. Prior to recording the concentrations, the spectrophotometer was blanked with the appropriate elution buffer.

2.7.3 Nucleic acid sequencing

Nucleic acid sequencing was performed on an ABI 3730 capillary sequencing instrument (Applied Biosystems, Warrington, UK) by the Gene Pool (Edinburgh University). DNA sequences were analysed using Chromas Lite software and translated into the amino acid sequence using Expasy Translate software.

2.8 DNA isolation

2.8.1 Genomic DNA isolation

Genomic DNA was isolated for the purpose of repetitive PCR analysis of strains. DNA was isolated using the Wizard Genomic DNA Isolation kit (Promega, Southampton, UK) according to the manufacturer's instructions. DNA preparations were stored at -20ºC.

2.8.2 Plasmid DNA isolation

Plasmid DNA was isolated using either the Qiagen Mini or Midi Prep kit according to the manufacturer's instructions. Plasmids were stored at -20ºC.

2.9 Pulsed-field gel electrophoresis (PFGE)

2.9.1 Agarose plugs

PFGE was performed using the modified protocol of Miranda *et al*. Strains were grown overnight in 5 ml of LB broth at 37°C and harvested by centrifugation at 3000rpm for 20 mins. The supernatant was discarded and the cells were resuspended in 2 ml of PIV buffer (1M Tris, 1M NaCl, pH7.6) and equilibrated in a waterbath to 50°C. Two ml of 1.6% Certified Megabase Agarose (BioRad, Hertfordshire, UK), melted in PIV buffer, was added to each cell suspension and the mixture was pipetted

into plug mould before being refrigerated at 4°C for 45 mins. The plugs were removed from the moulds and incubated in 5 ml of lysis buffer (1M Tris, 1M NaCl, 0.1M EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Sodium lauryol sarcosine, 50 mg of ribonuclease A per ml, and 1 mg of lysozyme [pH 7.6] per ml) overnight at 37°C. The lysis buffer was removed and replaced with 5 ml of ESP (0.4 M EDTA, 1% sodium lauroyl sarcosine [pH 9.0] and 0.5 mg of proteinase K per ml) buffer and plugs were incubated overnight at 50°C. The ESP buffer was removed and plugs were washed once with 1X TE (5 mM Tris, 5 mM EDTA [pH 7.5]) buffer and 3 times with 0.1x TE buffer for at least 30 mins each wash and at 37°C. Plugs were then stored in 0.5x TBE buffer (45mM tris, 45mM boric acid, 1mM EDTA, pH8.0) at 4°C until used.

2.9.2 Agarose plug digestion

Agarose plug slices of ~2mm were equilibriated at room temperature in 100µl of 1x restriction buffer for 30 mins. The restriction buffer was then replaced with fresh buffer containing 30U of *Xba*I and tubes were incubated in a waterbath at 37ºC for 18 hrs.

2.9.3 Gel preparation

A 1% Pulsed Field Certified Agarose (Bio-Rad, Hertfordshire, UK) gel was prepared in 0.5x TBE buffer. Plugs were loaded into the gel lanes which were then overlain with more 1% PFGE-grade agarose to seal.

2.9.4 Running conditions

Gels were run for 22 hours at 5.0 volts/cm with a pulse time of 5-35 seconds at 14ºC in 0.5x TBE running buffer, performed with a CHEF DRII system (Bio-Rad, Hertfordshire, UK). A Lambda Ladder PFG Marker (New England Biolabs Hertfordshire, UK) was used as a size standard.

2.9.5 Gel staining

Gels were stained in ethidium bromide as described previously and destained in distilled water when necessary. Gels were then visualised on the Bio-Rad GelDoc system.

2.9.6 S1 nuclease PFGE plasmid profiling

For the purpose of profiling larger plasmids, S1 nuclease (Promega, Hampshire, UK) was used to digest agarose plug slices that had been prepared as described previously for PFGE. Plug slices were equilibrated in 100µl of 1x S1 nuclease buffer at room temperature for 30 mins before being replaced with fresh 1x S1 nuclease buffer containing 8U of S1 nuclease. These were incubated in a waterbath for 45 mins at 37°C before reactions were terminated by the addition of 5µl of 0.5M EDTA. Gels were run and stained as described previously for PFGE.

2.10 Iso-electric focussing (IEF)

2.10.1 Preparation of cell lysates

Five ml of overnight cultures were harvested by centrifugation at 2000 g for 10 mins at 4°C. The cell pellets were washed with 5 ml of 50mM sodium phosphate buffer, pH7.0 and centrifuged as before. Cell pellets were resuspended in 2 ml of the sodium phosphate buffer and were sonicated, on ice, for 20 seconds at 8 microns four times with cooling periods in between pulses. Cell debris was cleared by centrifugation at 16,000 g for 5 mins at 4°C. The supernatant was stored at -20°C.

2.10.2 Assessment of β-lactamase activity

β-lactamase activity was assessed by measuring the time it took for 30µl of the preparation to change the colour of 100µl of a 50µg/ml nitrocephin solution.

2.10.3 Gel preparation

IEF gels were prepared containing a broad range of carrier ampholytes (pH3.5-10). The gel solution was prepared on glass plates as shown in Table 2.9. To promote adhesion one plate was first submerged in a binding solution (0.5% w/v gelatine) for 10 mins before being dried in a 55°C incubator for 20 mins. The other plate was siliconised to prevent adhesion. The plates were separated by rubber tubing along

their perimeters and placed in a casting chamber. The gel solution was poured and was left to polymerase in sunlight for 4 hours.

Component	Volume (ml)	Final Concentration
Ampholines	0.8	2% w/v
40% acrylamide	9	9%
Riboflavin (20mg/ml)	4	2mg/L
Distilled water	26	N/A
5% TEMED	0.2	0.005% (v/v)

Table 2.9. **The components and composition of the IEF gels.** N/A – not applicable

2.10.4 Running conditions

β-lactamase samples of roughly equivalent activity (as determined by the results of 2.10.2) were loaded on the gel surface close to the anode. The gels were run under the following conditions: voltage = 500V (limiting), current = 20mA (limiting), power = 1W (constant).

2.10.5 Band visualisation

To visualise β-lactamases a sheet of filter paper was soaked with a 50µg/ml nitrocephin solution which was then placed on the gel surface. Where the β-lactamases had focussed the filter paper would turn red.

2.11 Outer membrane protein (OMP) analysis

2.11.1 OMP extraction

OMPs were extracted according to the protocol as detailed by Bossi and Figueroa-Bossi (2007). Overnight cultures were inoculated 1/100 into 25mls of fresh LB broth in a sterile 125ml conical flask and shaken at 37°C, 180 rpm. Cultures were grown to mid-exponential phase (OD_{600} ~ 0.5) and 10 ml was pelleted by centrifugation at 4°C at 2000 g for 10 mins. The supernatant was discarded and pellets were resuspended in 1ml of 10mM Tris-HCl pH 8.0 and sonicated (MSE Soniprep 150) on ice twice for 15 seconds at 10 microns. Lysates were centrifuged for 5 mins at 4500 g at room temperature in order to remove cell debris. The supernatant was then removed to a 1.5 ml sterile microcentrifuge tube and centrifuged at 16,000 g for 45 mins at 20°C. Pellets were resuspended in 500µl of 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 2% (v/v) Triton X-100 and incubated in a waterbath at 37°C for 30 minutes. This was then centrifuged at 16,000 g for 45 mins at 20°C. The residual OMP pellet was resuspended in 50µl of 100mM Tris-HCl pH 8.0, 2% SDS. OMPs were stored at -20ºC.

2.11.2 Protein concentration estimation

Protein concentrations were estimated using a Pierce BCA Protein Assay kit (Thermo Scientific, Cramlington, UK) according to the manufacturer's instructions.

2.11.3 SDS PAGE gel casting

SDS PAGE gels were cast using the Bio-Rad Mini-PROTEAN® casting chambers. The components of the resolving and stacking gels are shown in table 2.10. The resolving gel was prepared first. Once poured the gel was quickly overlaid with 100% ethanol and allowed to polymerise for one hour. The ethanol was then poured off from the gel before the stacking layer was poured on top of the resolving gel and the comb inserted, ensuring a 0.5cm gap between the tips of the comb teeth and the resolving layer matrix. The stacking layer was allowed to polymerise for at least one hour.

	Resolving	**Stacking**
Gel Percentage	15	4
Component		
40% polyacrylamide (ml)	3.75	0.5
1.5M Tris-HCl, pH8.8 (ml)	2.5	N/A
1M Tris-HCl, pH6.8 (ml)	N/A	0.625
10% SDS (µl)	100	50
30% ammonium persulfate (µl)	20	10
TEMED (µl)	20	10
SDW (ml)	3.61	3.81
Total Volume (ml)	10	5

Table 2.10. **SDS PAGE gel components.**

2.11.4 SDS PAGE running conditions

SDS PAGE electrophoresis was performed using a Bio-Rad Mini-PROTEAN® II Cell. Samples were boiled in Laemmili buffer (Bio-Rad, Hertfordshire, UK) for 10 mins and chilled on ice prior to loading. Gels were run at 150V for ~2 hours in 1X tris-glycine SDS buffer (25mM tris, 192mM glycine, 0.1% SDS).

2.11.5 SDS PAGE gel staining

SDS PAGE gels were removed from the electrophoresis cell and briefly washed with distilled water before Coomassie staining and destaining. Stain/Destain; 50% ethanol, 7.5% acetic acid, 0.025% Coomassie R250 (stain solution only) (Bio-Rad, Hertfordshire UK). Gels were stained for ~30 mins and destained for 30-60mins.

2.12 Bacterial growth curves

Overnight cultures were inoculated 1/100 into 25mls of fresh LB broth in a sterile 125ml conical flask. Flasks were incubated at 37°C and shaken at 180 rpm. Optical density (OD) readings were taken in a spectrophotometer (Camspec M330) at a wavelength of 600nm at each hour.

2.13 Biofilm assays

Biofilm assays were performed according to the protocol of O'Toole and Kolter (1998). Overnight cultures were inoculated 1/100 into fresh LB broth. 100µl of each strain was pipetted into a 96-well polystyrene plate and plates were sealed. Plates were incubated at 37°C for 24, 48 and 72 hours. Controls of just LB broth were included. After the incubation period the contents of the wells were aspirated and each well was washed twice with sterile distilled water. 100µl of 0.1% crystal violet was added to each well and plates were incubated at room temperature for 15 minutes. The crystal violet was aspirated and wells were washed with sterile

distilled water twice as before. 125µl of 95% ethanol was added to each well to solubilise the crystal violet and transferred into a 1.2ml cuvette containing 875µl of distilled water. The contents of the cuvette were mixed and OD readings were taken in a spectrometer at a wavelength of 600nm. Assays were performed in triplicate. Readings taken from the LB broth only wells were used as background readings and subtracted from the subsequent calculations of the levels of biofilm production.

2.14 Mutation studies

2.14.1 Attainment of mutants

Overnight cultures were serially diluted into sterile saline ranging from approximately 10^9 -10^3cfu/ml and spread on IST only plates and IST plates containing the appropriate antimicrobial or compound at 2, 4 and 8 times their respective MICs. Plates were incubated in air at 37°C overnight. Colonies were picked from the antibiotic containing plates and inoculated into fresh LB broth. Mutants were subjected to susceptibility testing and freezer stocks were prepared to be stored at -80ºC. Colony counts were performed on the IST only plates in order to calculate mutation frequencies.

2.14.2 Stability of mutants

To test the stability of the phenotypes of the mutant strains, each was subcultured on IST agar plates for 10 days before being subjected to susceptibility testing. Those

showing the same susceptibility profile as the original mutants were considered to have a stable phenotype.

2.15 Gene expression induction studies

2.15.1 Chlorpromazine

Bacterial cultures were seeded (1/100) into fresh LB broth and grown shaking at 37ºC. At mid-exponential growth phase (OD_{600} 0.4-0.6) the cultures were split; 150µg/ml CPZ was added to one half of the culture and the other half remained untreated. These were then grown shaking at 37ºC for a further hour prior to cells being harvested for RNA extraction.

2.15.2 Tigecycline

Bacterial cultures were seeded (1/100) into fresh LB broth and grown shaking at 37ºC. At mid-exponential growth phase (OD_{600} 0.4-0.6) the cultures were split; tigecycline was added to one half of the culture at 4 times the respective MIC of the strain and the other half remained untreated. These were then grown shaking at 37ºC for a further hour prior to cells being harvested for RNA extraction.

2.16 Gene expression analysis

2.16.1 RNA extraction

RNA was extracted using the RiboPure Bacteria RNA kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions and RNA was treated with TURBO DNA-*free* DNase (Applied Biosystems, Warrington, UK) to eliminate any contaminating DNA. One µl of DNase digested RNA from each sample was used as a template for PCR amplification using primers RamAF and RamAR for 30 cycles. If amplification was observed, TURBO DNA-*free* DNase treatment was repeated. RNA was run on an agarose gel in order to assess integrity and check for degradation. RNA was stored at -80°C.

2.16.2 RNA concentration estimation

The concentration of extracted RNA samples was estimated using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Cramlington, UK). Two µl of each RNA sample was read by the spectrophotometer and 260/280 absorbance readings were noted. Those that fell between 1.8 and 2.1 were deemed to suitable to be carried forward for cDNA synthesis.

2.16.3 cDNA synthesis

cDNA synthesis was carried as detailed in manufacturer's instructions using the Invitrogen First Strand cDNA Synthesis kit. (Invitrogen, Paisley, UK) Briefly, 250ng of total RNA, 500µM dNTPs and 50ng of random hexamers were incubated at 65ºC for 5 min then chilled on ice. Reverse transcription was carried out in a total volume of 20µl containing; 10mM DTT, 5mM $MgCl_2$, 1X RT buffer, 40U of RNaseOUT and 200U of SuperScript III reverse transcriptase, for 10 min at 25ºC, 50 min at 50ºC and the reaction was terminated by heating for 5 min at 85ºC.

2.16.4 Reverse transcription (RT) PCR

RT PCR was performed on genes of interest. PCRs for all genes were performed using 1µl of cDNA in a 50µl total volume for 25-30 cycles, depending upon the gene, with the exception of the 16S gene, which was for 12 cycles (Table 2.11).. Table 2.12 shows the genes for which expression was measured in the investigated strains. *K. pneumoniae* ATCC 13883 genomic DNA was also included as a PCR positive control.

Gene	PCR Cycles
ompK34	28
ompK35	28
ompK36	28
ramA	28
romA	28
ramR	30
ybdJ	28
acrA	25
16S	12

Table 2.11. **Cycle numbers used for PCR assessment of each gene.**

***K. pneumoniae* Strain**	**Genes**
K1	*ompK34, ompK35, ompK36, ramA, romA, acrA, 16S*
K2	*ompK34, ompK35, ompK36, ramA, romA, acrA, 16S*
ATCC 13883	*ompK34, ompK35, ompK36, ramA, romA, acrA, 16S*
Ecl8	*ramA, ramR, romA, acrA, ybdJ, 16S*
Ecl8 Mdr1	*ramA, ramR, romA, acrA, ybdJ, 16S*
S7	*ramA, ramR, romA, acrA, ybdJ, 16S*
S8	*ramA, ramR, romA, acrA, ybdJ, 16S*
CG43	*ramA, ramR, romA, acrA, ybdJ, 16S*
MGH 78578	*ramA, ramR, romA, acrA, ybdJ, 16S*
Kp342	*ramA, ramR, romA, acrA, ybdJ, 16S*
S28	*ramA, ramR, romA, acrA, ybdJ, 16S*
S29	*ramA, ramR, romA, acrA, ybdJ, 16S*

Table 2.12. **The *K. pneumoniae* strains on which RT-PCR was performed, the genes assessed for each strain, and cycles used for each assessment.**

2.16.5 Gel analysis

RT PCR products were analysed using the Bio-Rad GelDoc 2000 software, Quantity One. After being run on agarose gels and staining, Quantity One was used to semi-quantitatively assess gene expression. The intensities of the PCR product bands were measured and normalised to the intensities of the 16S bands before being used to compare expression levels between strains. The following settings and conditions were used: manual band selection, background subtracted, peak intensity readings measured, rolling disc value of 10 used.

2.17 Plasmid curing experiments

2.17.1 Acriflavine, SDS and ethidium bromide

Overnight broth cultures were inoculated into LB broth containing either doubling dilutions of ethidium bromide or 256µg/ml of acriflavine or SDS. These were then grown shaking at 180 rpm, overnight at 37°C. For ethidium bromide cultures aliquots of the dilution in which growth appeared to be visibly inhibited, 128µg/ml, was spread onto IST agar plates. Aliquots of acriflavine and SDS cultures were similarly spread on IST agar plates. All plates were incubated in air at 37°C for 18 hours. Twenty colonies were picked for each strain and each curing agent, inoculated into LB broth for growth overnight, and their susceptibility tested against ertapenem, imipenem and meropenem. If strains showed similar susceptibility profiles as the parents then it was deemed that no plasmid effecting carbapenem

resistance was lost. If strains showed reduced MICs then it was deemed that it was likely that a plasmid containing a β-lactamase had been lost and warranted further investigation.

2.18 Genetic manipulations

2.18.1 Preparation of chemically competent DH5α cells

An overnight broth culture of *E. coli* DH5α was inoculated 1/100 into 50mls of fresh LB broth in a sterile 250ml flask. This was then shaken at 37°C at 180rpm until the culture reached an OD_{600} of approximately 0.5. The culture was split into two 25ml volumes in sterile 50 ml centrifuge tubes and the cells were pelleted by centrifugation at 4°C at 2000 g or 10mins. The supernatant was removed and the cell pellets were resuspended in 25mls of sterile ice cold 0.1M $CaCl_2$ then placed on ice for 30mins. The tubes were centrifuged as before at 4°C at 2000 g for 10mins. The supernatant was removed and the cell pellet was resuspended in 1ml of sterile ice cold 0.1M $CaCl_2$ to which 200µl of 90% glycerol was added. The cells were stored as 100µl aliquots at -80°C.

2.18.2 Preparation of electrocompetent cells

An overnight broth culture was inoculated 1/100 into 50mls of fresh LB broth in a sterile 250ml flask. This was then shaken at 37°C at 180rpm until the culture reached an OD_{600} of approximately 0.5. The culture was split into two 25ml volumes

in sterile 50ml centrifuge tubes and placed on ice for 30 mins. Cells were pelleted by centrifugation at 4°C at 2000 g for 10 mins. The supernatant was removed and the cell pellets were resuspended in 25mls of sterile ice cold 10% glycerol before being centrifuged as before. This step was repeated twice more with the pellet being resuspended in reducing volumes of glycerol (15mls then 10mls). The final pellet was resuspended in 1ml of sterile ice cold 10% glycerol and cells were stored as 100µl aliquots at -80°C.

2.18.3 Plasmid transformation via heatshock

An aliquot of chemically competent DH5α cells were thawed on ice. Two µl of purified plasmid or 5µl of ligation was added to the cells, mixed and chilled on ice for 30mins. Cells were then placed in a waterbath set to 42°C for exactly 1 minute then immediately placed on ice for 2 mins. One ml of fresh LB broth was added to the cells which were then transferred into a sterile 15 ml centrifuge tube and incubated at 37°C, shaking at 180rpm for 1 hour. Cells were then spread on LB agar plates containing the appropriate selective antibiotics and incubated overnight at 37°C.

2.18.4 Plasmid transformation via electroporation

An aliquot of electrocompetent cells were thawed on ice. One µl of plasmid was pipetted into a 2 mm gap electroporation cuvette (Invitrogen, Paisley, UK). Cells were added to the cuvette and briefly mixed before being placed back on ice. Cells

were electroporated at 2.5kV in a Bio-Rad Micropulser and 1 ml of LB broth was immediately added to the cells. The cells were then transferred in a sterile 15 ml centrifuge tube and incubated at 37°C, shaking at 180 rpm for 1 hour. Cells were then spread on LB agar plates containing the appropriate selective antibiotics.

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2.18.5 *ramR* cloning and complementation

The entire *ramR* open reading frame (ORF) was previously amplified using primers RamRF and RamRR, and cloned into vector pACYC177 (by T. Schneiders), subsequently known as pAC*ramR*. Both pACYC177 and pAC*ramR* were maintained in *E. coli* DH5α. pAC*ramR* and pACYC177 were transformed into electrocompetent Kp342 cells by electroporation and selected for using kanamycin at 30µg/ml. Plasmids were confirmed by performing plasmid preparations and subsequent restriction analysis. Those containing pACYC177 only were restricted with *Sca*I to linearise and give a single band of approximately 3.9 kb. Those containing pAC*ramR* were restricted with both *Sca*I and *Pst*I to give two bands of approximately 3.6 kb and 0.9 kb. *ramR* presence was additionally confirmed by PCR using primers internal to *ramR*. Upon confirmation the strains were frozen down and stored at -80°C for further work.

2.18.6 *romA* cloning and complementation

romA was amplified from *K. pneumoniae* MGH 78578 with the primers RomAF2 and RomAR2, and using the Stratagene Easy-A Cloning enzyme. The PCR product

was cloned into the pGem T-easy plasmid using the pGem T-easy vector system (Promega, Hampshire UK), according to the manufacturer's instructions. *romA* was cut from the pGem plasmid using restriction enzymes *Sca*I and *Pst*I, and ligated into pACYC177, also cut with the same enzymes. The ligation was transformed via heat shock into *E. coli* DH5α cells. After confirmation of the transformation by plasmid preparation and restriction in the same manner as described previously for *ramR*, the pAC*romA* plasmid was extracted using a Qiagen Mini Prep kit and transformed into electrocompetent cells of *E. coli* MG1655 and also *K. pneumoniae* Ecl8, by electroporation. The transformation was again confirmed by plasmid extraction and restriction analysis. Strains were frozen down and stored at -80°C for further work.

2.19 Phenotypic β-lactamase detection assays

To detect the presence of any AmpC β-lactamases and any carbapenemases, the following tests were performed: a modified three dimensional test, an AmpC disc test and a modified Hodge test.

2.19.1 The modified three dimensional test

Crude enzyme extracts were prepared for strains K1 and K2 as described previously for IEF. A lawn culture of *E. coli* ATCC 25922 was prepared on Mueller Hinton (MH) agar plates. A cefoxitin disc was placed in the centre of the plate and using a sterile scalpel, 3cm linear slits were cut 3mm away from the disc. At the end of the slit, away from the disc, a small circular well was cut in the agar to which 40µl of

enzyme extract was loaded. Plates were allowed to dry for 10 minutes before being incubated in air at 37°C for 24 hours. Distortion of the zone of inhibition across the slits would be indicative of cefoxitin hydrolysis and possible AmpC production. Strains giving no visible distortion are considered non-AmpC producers.

2.19.2 AmpC disc test

A lawn culture of *E. coli* ATCC 25922 was prepared on MH agar plates. A cefoxitin disc was placed in the centre of the plate. Sterile filter paper discs (6mm) were moistened with 5µl of sterile saline and inoculated with several colonies of the test organism. The inoculated disc was then placed next to the cefoxitin disc without touching. A positive test for AmpC production appeared as a distortion or indentation of the zone of inhibition in the vicinity of the test disc.

2.19.3 Modified Hodge test

Modified Hodge tests were performed according to the protocol of Lee *et al* (2001). *E. coli* 25922 was used as the indicator strain and *K. pneumoniae* MGH 78578 was used as the carbapenemase –ve strain. MH agar plates were swabbed with a suspension of *E. coli* 25922 diluted in sterile saline to the equivalent of a 0.5M McFarland standard. After plates had dried a test disc, either ertpanem, imipenem or meropenem, was placed in the centre of the plate. A heavy inoculum of test strain was then streaked from the disc, without touching, to the edge of the plate. This was performed with strain K1, K2, *K. pneumoniae* MGH 78578 (carbapenemase –ve

control), and *K. pneumoniae* KPC-3 (carbapenemase +ve control). Plates were the incubated in air at 37°C for 18 hours. If a cloverleaf effect was observed with the indicator strain then it was concluded that a carbapenemase was likely to be present in the test strain where this effect was observed.

2.20 Transconjugation studies

2.20.1 Transconjugation assays

Transconjugation assays were performed as follows: overnight broth cultures of the recipient *(E. coli* J62-2) and donor strains (K1 and K2) were mixed in a 1:4 ratio, cells were collected by centrifugation and resuspended in 30µl of cold saline. 5 µl aliquots of the resuspension were spotted onto a nutrient agar plate and incubated at 37°C for 6h. Growth was collected and resuspended in cold saline and inoculated onto nutrient agar plates containing rifampicin at 16mg/L and one of the selective antibiotics gentamicin, ceftazidime or meropenem at a range of concentrations.

2.20.2 Assessment of transconjugants

Selected putative transconjugant strains were assessed by susceptibility testing and S1 PFGE.

Chapter 3. Chemical induction of the Ram regulon

3.1 Introduction

3.1.1 The RamA regulon

Although the transcriptional activator RamA was first identified in *K. pneumoniae* in 1995 (George *et al*, 1995), its exact role in conferring antibiotic resistance and the scope of its regulon continue to remain elusive. Previous studies have associated *ramA* over-expression with an increase in efflux, specifically the increased expression of the AcrAB efflux pump; however it is unknown whether this is an exclusive relationship and how this effect is activated (Kallman *et al*, 2008; Zheng *et al*, 2009). It is known that mutations within *acrR*, the gene encoding a local repressor of the AcrAB efflux pump, can result in the over-expression of the pump and lead to an MDR phenotype (Olliver *et al*, 2004). However MDR phenotypes as a result of *acrAB* over-expression are also evident in the absence of *acrR* mutations, leading to the assumption that other regulatory mechanisms are in place, such as the contribution of RamA (Schneiders *et al*, 2003). This study looks at the ability of two compounds, the glycylcycline antibiotic tigecycline and the phenothiazine compound chlorpromazine (CPZ), in activating the expression of *ramA* and members of its putative regulon in a panel of *K. pneumoniae* strains.

3.1.2 Chlorpromazine background

Chlorpromazine is an aliphatic phenothiazine antipsychotic drug initially used in the treatment of schizophrenia in the 1950's (Figure 3.1) (Owens, 1996). It acts as an antagonist of various postsynaptic receptors within the central nervous system (dopamine, serotonin, histamine, adrenergic and muscarinic acetylcholine receptors), however whether the antagonism is caused by the direct binding of chlorpromazine to the receptors or as an indirect effect is unknown (Owens, 1996). Chlorpromazine has also been used in the treatment of nausea/emesis, anxiety and amphetamine overdose (Owens, 1996).

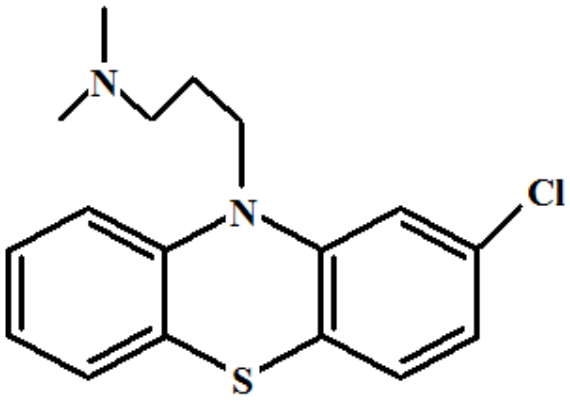


Figure 3.1. **The aliphatic side chain of chlorpromazine.** The three ring structure, with nitrogen and sulphur, is the defining characteristic of a phenothiazine compound. (adapted from Bourdon, 1961)

Chlorpromazine has been noted in several studies (Amaral *et al*, 2000; Kaatz *et al*, 2003; Chan *et al*, 2007) for its antimicrobial effects but unfortunately its potential for

use in the treatment of bacterial infections is severely limited by the severity and toxicity of its side effects and the high concentrations required to exert its antimicrobial activity. Studies in *S. aureus* and *Burkholderia pseudomallei* have shown that chlorpromazine can act synergistically with certain antibiotics to lower their respective MICs (Kaatz *et al,* 2003; Chan *et al*, 2007), although the exact mechanism of this synergy is still unknown. Chlorpromazine itself is suspected to act via the binding of calcium binding proteins, inhibiting calcium-dependent enzymatic processes and thereby impeding the proton gradient-dependent efflux of toxic compounds (Weiss *et al*, 1980). It is because of this hypothesis that chlorpromazine may be referred to as an efflux pump inhibitor (EPI) in some scientific publications, although it is important to emphasise that this has not yet been confirmed experimentally.

Chlorpromazine was chosen for study in *K. pneumoniae* because of evidence presented in a previous study in *S. enterica* that suggested chlorpromazine could upregulate *ramA* expression. Bailey *et al* (2008) found that chlorpromazine independent *ramA* over-expression resulted in *acrB* over-expression but in contrast, chlorpromazine dependent *ramA* over-expression resulted in *acrB* downregulation. It was concluded that chlorpromazine acted on *acrB* independently of *ramA* and subsequently the regulation of both genes following chlorpromazine exposure was deemed not to be linked. Whether this effect is also true in *K. pneumoniae* remains to be determined.

3.1.3 Tigecycline background

Tigecycline is a semisynthetic glycylcycline antibiotic, marketed by Wyeth as Tygacil®, that was approved for clinical use in 2005. Tigecycline is actually a derivative of the tetracycline antibiotic, minocycline, and is currently the sole member of the glycylcycline class of antibiotics licensed for clinical use. The glycylcyclines were initially developed in response to the increasing incidence of MDR bacterial infections and were designed to overcome mechanisms of resistance responsible for rendering other antimicrobials ineffective. Similarly to the tetracyclines, tigecycline is a bacteriostatic protein synthesis inhibitor that functions by binding the 30S ribosomal subunit and blocking the entry of amino-acyl tRNA to the A site of the ribosome. The substitution of an N-alkyl-glycylamido group at the 9 position on the D ring allows tigecycline to evade the normal resistance mechanisms, such as the *tet* transporters, to which tetracycline is vulnerable (Noskin, 2005) (Figure 3.2).

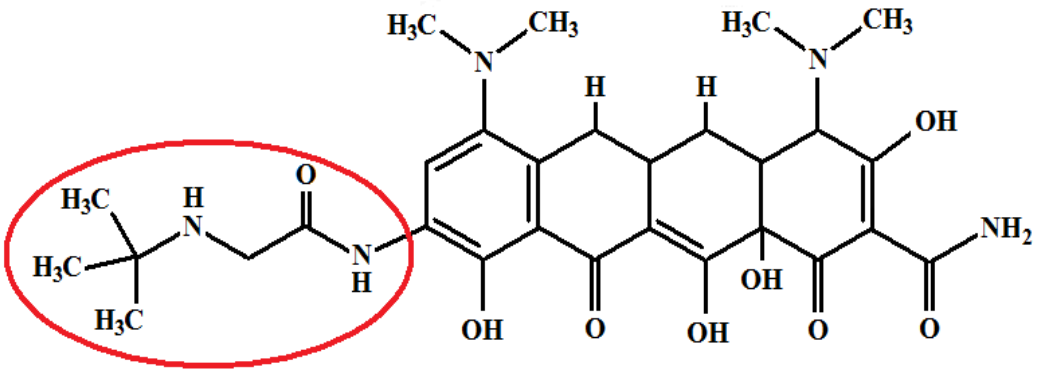


Figure 3.2. **The primary structure of tigecycline.** The N-alkyl-glycylamido group is circled in red. (adapted from Noskin, 2005)

Tigecycline has been shown to have broad spectrum activity against Gram-negative, Gram-positive and anaerobic bacteria (Noskin, 2005). Specifically, tigecycline has exhibited significant potency against particularly problematic infection causing bacteria such as MRSA and MDR *A. baumannii* (Noskin, 2005).

Tigecycline was chosen for analysis in this study due to a reported correlation between *ramA* over-expressing strains and tigecycline resistance (Ruzin *et al*, 2005).

3.2 Results

3.2.1 Susceptibility testing

The panel of *K. pneumoniae* strains were subjected to susceptibility testing against antibiotics from four different classes; chloramphenicol, fluoroquinolones (ciprofloxacin and norfloxacin), tetracyclines (minocycline and tetracycline) and glycylcyclines (tigecycline). The susceptibility testing results, as shown in Table 3.1, indicate that the strains listed as *ramA* over-expressers have significantly higher MICs to the antibiotics tested than the *ramA* non-expressing strains with the only exception being the MIC of the fluoroquinolones, ciprofloxacin and norfloxacin, for Kp342, which is comparable to that of the non-expressing strains. These results suggest that the expression of *ramA* plays a role in conferring resistance to these antibiotics. Note: it has previously been determined by T. Schneiders that the RamA and AcrR protein sequences are identical for all strains tested (data not shown).

	MIC (µg/ml)						
	Cip	**Nor**	**Cm**	**Mino**	**Tet**	**Tige**	
Range Tested	0.0625-512	0.0625-512	0.0625-512	0.0625-32	0.0625-512	0.0625-16	
Strain							***ramA* Status**
S7	8	>128	>512	>32	>512	8	Expresser
S8	0.5	4	4	4	2	0.125	Non-expresser
Ecl8	<0.0625	<0.0625	0.125	2	0.25	0.125	Non-expresser
Ecl8 Mdr1	8	128	128	32	256	4	Expresser
Kp342	0.5	4	256	>32	16	4	Expresser
MGH 78578	1	4	>512	>32	512	0.25	Non-expresser
S28	32	32	>512	>32	>512	2	Expresser
S29	64	256	>512	>32	>512	2	Expresser
CG43	<0.0625	0.125	2	8	1	0.5	Non-expresser

Table 3.1. **The resistance profiles of strains investigated their *ramA* expression status.**

* *ramA* expression status was previously determined via northern blot analysis by T. Schneiders: data not shown.

3.2.2 Chlorpromazine and tigecycline studies

The effects of chlorpromazine, a reported EPI was investigated in its capacity to inhibit active efflux, and both chlorpromazine and tigecycline were examined in their effects on the expression of *ramA* and its related genes.

3.2.3 Synergy testing

MDR *ramA* over-expressing strains were chosen for synergy testing with chlorpromazine and three structurally unrelated antibiotics that are known from previous literature to be efflux pump substrates; chloramphenicol (Cm), norfloxacin (Nor) and tetracycline (Tet) (Elkins and Nikaido, 2002). Chlorpromazine was shown to act synergistically in all strains tested when used at a set concentration of 200µg/ml (<50% of the MIC value for each strain) in combination with norfloxacin (Nor) (Table 3.2). FIC values were determined and synergy was defined as a FIC value of ≤0.5. Synergy with chloramphenicol and tetracycline appeared to be more strain dependent, with two and four strains showing synergy respectively. Of the strains that did not show synergy, an additive effect was observed (Addition= FIC >0.5-1). Overall, the use of chlorpromazine resulted in reductions in the MICs ranging from 4 – 128-fold. These results suggest that chlorpromazine has an inhibitory effect on the efflux systems and that active efflux is at least partly responsible for the resistance phenotypes displayed by these strains to the antibiotics tested.

Strain	CPZ	Cm	Cm/CPZ_{200}	FIC*	Fold Decrease	Nor	Nor/CPZ_{200}	FIC*	Fold Decrease	Tet	Tet/CPZ_{200}	FIC*	Fold Decrease
S7	512	2048	512	0.9	4	2048	16	0.4	128	1024	256	0.6	4
Ecl8 Mdr1	512	128	8	0.5	16	8	0.25	0.4	32	4	0.25	0.5	16
Kp342	1024	256	64	0.4	4	4	1	0.4	4	16	4	0.4	4
S29	512	2048	512	0.9	4	256	4	0.4	64	512	64	0.5	8
S28	512	2048	512	0.9	4	32	4	0.5	8	1024	64	0.5	16

Table 3.2. **Chlorpromazine synergy MICs.** MIC values for tested strains with antibiotic alone and in combination. FIC values and fold decreases in MIC are also shown for the testing combinations. Values in red are deemed to show synergy.

*Synergy was defined as combinations showing an FIC≤0.5. MIC values are in µg/ml.

3.2.4 Chlorpromazine transcriptional induction of the Ram regulon

Exponential growth phase cultures of a panel of *ramA* non-expressing *K. pneumoniae* strains (S8, Ecl8, MGH 78578 and CG43) were split; one half of each culture was left untreated and the other half was treated with 150µg/ml of chlorpormazine, before both were grown shaking at 37ºC for one further hour. RNA was extracted and cDNA synthesised in order to perform RT-PCR and assess the transcription of genes *ramA*, *romA*, *acrA, ramR* and *16S*. The cultures treated with chlorpromazine were shown to exhibit increased transcription levels of *ramA*, *romA* and *acrA* (Figure 3.3.). No *ramR* transcript could be detected in either culture in any of the strains.

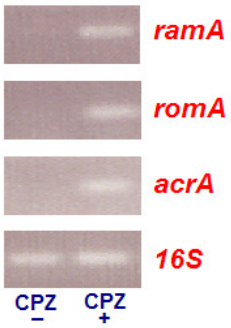
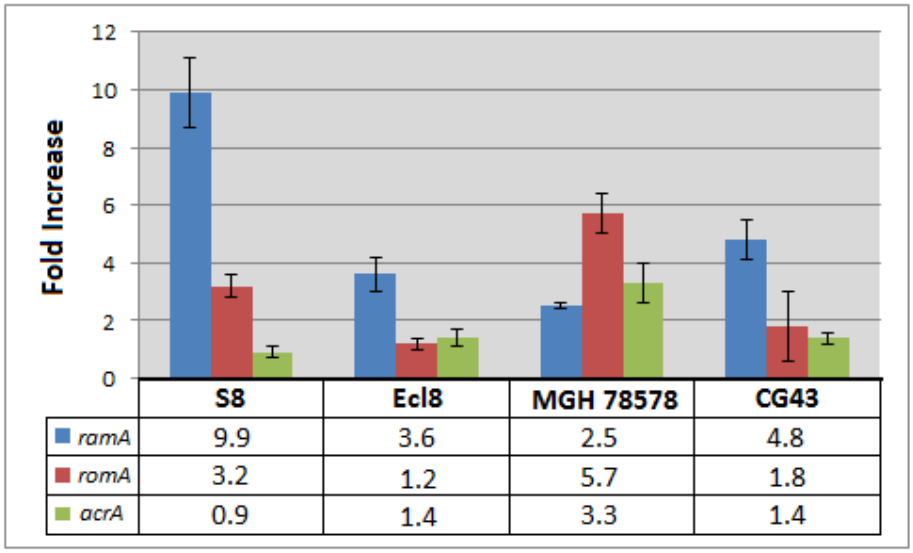


Figure 3.3. **Example gel showing chlorpromazine induction in strain MGH 78578.** An agarose gel picture showing an example of the transcriptional induction of genes *ramA*, *romA* and *acrA* by chlorpromazine in strain MGH 78578 after one hour of chlorpromazine treatment at 200µg/ml. - = untreated culture; + = chlorpromazine treated culture. *16S* is shown as an internal control.

Of the panel of strains treated with chlorpromazine, all appeared to exhibit the same effect of increased transcription of *ramA*, *romA* and *acrA*, albeit to varying levels between strains. The levels of transcription were quantified using the GelDoc Quantity One software (BioRad) as shown in Figure 3.4. In all strains, with the exception of MGH 78578, *ramA* transcription shows the greatest increase, followed by *romA* and then *acrA*. Note: all transcriptional analyses are semi-quantitative and although background fluorescence is subtracted prior to quantification, a margin of error remains. All RT-PCR analyses were performed twice on separate occasions.



	S8	Ecl8	MGH 78578	CG43
ramA	9.9	3.6	2.5	4.8
romA	3.2	1.2	5.7	1.8
acrA	0.9	1.4	3.3	1.4

Figure 3.4. **Graph showing the levels of chlorpromazine induction of genes *ramA*, *romA* and *acrA*.** Shows the fold differences in transcription levels between *ramA*, *romA* and *acrA* following CPZ treatment; all levels were normalised to *16S* and are relative to transcription levels detected in untreated cultures. The data table shows levels of expression as graphed. Error bars show standard eror of the mean (SEM).

3.2.5 Tigecycline transcriptional induction of the Ram regulon

Two *ramA* non-expressing *K. pneumoniae* strains, S8 and Ecl8, were chosen for tigecycline studies. Exponential growth phase cultures were split in two; one half was left untreated and the other treated with 8X the MIC of tigecycline (Table 3.3) before being grown shaking at 37ºC for two further hours. RNA was extracted and cDNA synthesised in order to perform RT-PCR.

	MIC (µg/ml)	
Strain	**Tig**	**8X**
S8	0.125	1
Ecl8	0.125	1

Table 3.3. **S8 and Ecl8 tigecycline MICs.** Shows the MIC for tigecycline for strains Ecl8 and S8 and the concentrations at which the inductions were performed.

RT-PCR was performed for genes *ramA*, *romA*, *acrA*, *ramR, ybdJ* and 16S rRNA respectively. Both strains S8 and Ecl8 showed increased transcription of genes *ramA*, *romA* and *acrA* following exposure to tigecycline (Figure 3.5). No *ramR* or *ybdJ* transcript was detected in either strain and under either growth condition. The transcription levels were quantified using GelDoc Quantity One software (BioRad) as before (Figure 3.6).

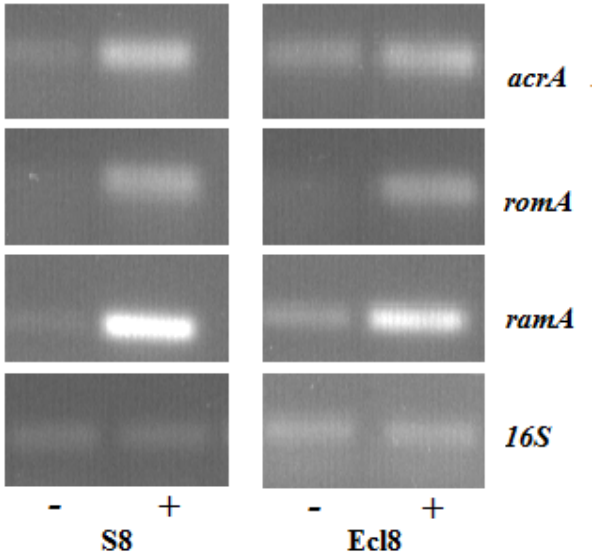


Figure 3.5. **Example gel showing tigecycline induction in strains S8 and Ecl8.** An agarose gel picture showing the effect of tigecycline on *ramA*, *romA* and *acrA* after two hours of exposure to tigecycline at 8x their respective MICs in strains S8 and Ecl8. - = untreated culture; + = tigecycline treated culture.

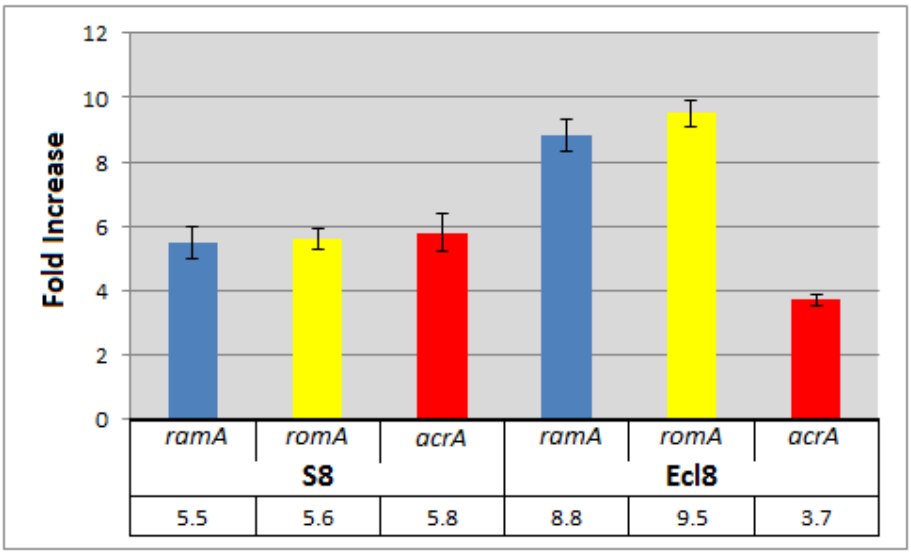


Figure 3.6. **Graph showing the levels of tigecycine induction of *ramA*, *romA* and *acrA*.** Shows the increases in transcription level of two strains, S8 and Ecl8, after exposure to tigecycline for two hours. All increases are relative to the transcription levels in untreated cultures and normalised to *16S* levels. Error bars show SEM.

As Figures 3.5 and 3.6 show, the transcription of *ramA*, *romA* and *acrA* was upregulated in both S8 and Ecl8 following exposure to tigecycline. The upregulation of all three genes was very similar in strains S8, whilst *acrA* was upregulated to a much lesser extent than that of *ramA* and *romA* in strain Ecl8. All RT-PCR analyses were performed twice on separate occasions.

3.3 Discussion

The transcriptional activator RamA has been shown in previous studies (Schneiders *et al*, 2003; Kallman *et al*, 2008; Zheng *et al*, 2009) to be involved in the regulation of the RND family efflux pump, AcrAB, in *K. pneumoniae* and *Salmonella* spp. The over-expression of such an efflux mechanism is sufficient to confer an MDR phenotype (Schneiders *et al*, 2003). AcrAB, for example, is capable of the efflux of several classes of antibiotics including the fluoroquinolones and the tetracyclines (Elkins and Nikaido, 2002). The consequence of this resistance mechanism is a severe limitation in treatment options for infections caused by such organisms and subsequently alternative antibiotic treatment regimens must be utilised.

In the current antibiotic climate, where few new compounds are being discovered or developed, it is increasingly important that upon the potential release of such compounds for clinical use that it is anticipated how resistance is likely to arise. Therefore, with tigecycline for example, it is important to anticipate how resistance may be conferred. The results of this may influence how the antibiotic is used clinically and which interventions are necessary in order to limit the development of resistance. In cases where MDR and even PDR strains are becoming more common, clinicians and scientists are now finding it necessary to obtain alternative treatments to the antibiotics typically used in the clinical setting (Falagas *et al*, 2008). This emergence has resulted in the revival of the polymyxins for the treatment of PDR strains (Falagas and Kasiakou, 2005) and has also prompted research into other

compounds, such as the phenothiazines, as a possible supplement to the current antibiotic compilation (Kaatz *et al*, 2003).

The susceptibility testing results showed that, as suggested in previous literature (George *et al*, 1995), the over-expression of *ramA* can be correlated with an MDR phenotype as shown by the elevated MICs of chloramphenicol, the fluoroquinolones, the tetracyclines and the glycylcycline. The only exceptions to this trait are the fluoroquinolones, (ciprofloxacin and norfloxacin), MICs exhibited by Kp342, which were comparable to those exhibited by the *ramA* non-expressing strains S8 and MGH 78578. This effect could be for a number of reasons, for example; the other *ramA* over-expressers may carry additional mutations that contribute to fluoroquinolone resistance such as *gyrA, gyrB*, *parC,* or *parE* mutations that Kp342 does not, or alternatively there may be mutations present within the efflux genes of Kp342 that can result in the fluoroquinolones being a poor substrate for the pumps.

The synergy testing results suggest that chlorpromazine may indeed be capable of functioning as an efflux pump inhibitor as has been suggested previously (Kaatz *et al*, 2003; Chan *et al*, 2007), however this effect does not appear to be consistent between strains and shows a variation in efficacy between antibiotics. All of the *ramA* over-expressing strain tested with chlorpromazine and norfloxacin gave values that could be interpreted as showing a synergistic effect, and similar results were observed for chlorpromazine and tetracycline, with the exception of S7 whose FIC value fell into the addition category. The greatest variation between strains was observed with chlorpromazine and chloramphenicol where only the FIC values of

Ecl8 Mdr1 and Kp342 fell into the synergy category, and the remaining strains showed addition. This effect could be due to other efflux systems or other chloramphenicol resistance mechanisms at work, which are not affected by chlorpromazine. However, if we only take the fold decreases into account, rather than the FIC values, then the use of chlorpromazine results in respectable MIC decreases ranging from 4-128-fold across all antibiotics and strains tested.

It appears that both chlorpromazine and tigecycline are capable of inducing the transcription of *ramA* and its associated genes, *romA* and *acrA*, as shown by the RT-PCR studies. Induction by chlorpromazine was exhibited in all *ramA* non-expressing strains and induction by tigecycline was exhibited in the two candidate strains Ecl8 and S8. Although all of the genes in question did indeed appear to be upregulated, the levels varied vastly between strains, suggesting that the regulation of these genes is a strain-dependent effect in which other unknown factors are involved. The similar upregulatory effect suggests that both compounds, chlorpromazine and tigecycline, may be capable of selecting for a MDR phenotype via the induction of *ramA* expression and subsequently *acrAB* upregulation. The results of this part of the study contrast those found by Bailey *et al* (2008). They found that whilst chlorpromazine upregulated *ramA*, they also determined that *acrB* was downregulated. Although in this study we looked at the expression of *acrA* rather than *acrB*, both genes are reported to be co-transcribed and so the same transcriptional effect could be expected in both genes (Keeney *et al*, 2008). There could be a number of reasons for this contrast in results. The study by Bailey *et al* (2008) was performed in *S. enterica* and so the observed effect may have been

species-specific or alternatively chlorpromazine may effect the transcription of *acrA* and *acrB* differently. It is possible that under certain conditions that, despite the co-transcription of *acrA* and *acrB,* the stability of their mRNA may differ or be affected by chlorpromazine, resulting in the apparent up or downregulation of either gene. However, it remains unclear from this data whether the apparent induction of expression is due to true upregulation of RNA transcription or an mRNA stabilisation effect, and additionally whether this leads to increased translation.

The lack of detection of both *ramR* and *ybdJ* by RT-PCR could be for a number of reasons, for example the genes may be subject to low level expression that is not picked up by RT-PCR analysis or that their expression could simply be repressed. Neither chemical caused the over-expression of these genes indicating that their expression is not directly affected by tigecycline or chlorpromazine in the same manner as was observed for *ramA*, *romA* and *acrA*. Previous reports indicate that RamR is involved in the regulation of the *ram* regulon (Bailey et al, 2008; Molitor, 2008), however *ybdJ* has not been previously reported to play a role in this regulon and the lack of induced over-expression indicates that this may indeed be the case.

The first discovered phenothiazine, methylene blue, was initially demonstrated to exhibit activity against *Plasmodium falciparum* by Guttmann and Erlich in the late 19th century (Guttmann and Ehrlich, 1891). Following this the first neuroleptic phenothiazine, chlorpromazine, was developed in the 1950's and was quickly shown to exhibit antimicrobial activity against *M. tuberculosis* (Bourdon, 1961), however the severe side-effects of chlorpromazine observed in patients treated long term for

schizophrenia resulted in no further development of chlorpromazine as an antimicrobial drug (Amaral *et al*, 2001). Chlorpromazine appears to be unsuitable for clinical use as even the lowest concentrations at which significant antimicrobial activity is exhibited are unachievable due to its toxic side effects (Amaral *et al*, 2001). However, in the current circumstances where an increasing number of PDR bacterial strains are being isolated (Falagas *et al*, 2008), the antimicrobial properties of phenothiazines indicate an attractive potential for their development and utilisation. However, the exact nature of their interactions both within the bacterial cell and the human body must first be elucidated in order to achieve optimal efficacy and safety. Although chlorpromazine has been shown to exhibit high levels of activity against *M. tuberculosis*, it is also amongst the most toxic of the phenothiazines. Thioridazine, a much milder neuroleptic phenothiazine with fewer side-effects compared to chlorpromazine, has been shown to exhibit identical *in vitro* levels of activity to that of chlorpromazine in *M. tuberculosis* (Amaral *et al*, 1996). Another attractive property of the phenothiazines is that, since they are proposed to function by the irreversible binding of calcium binding proteins, no resistance development is anticipated. Any gene deletions or mutations within the target proteins are likely to be lethal to the bacterial cell, so theoretically this should reduce the likelihood of the emergence of resistant strains (Amaral *et al*, 2001). Whilst the synergy testing results shown in this study indicate a potential for phenothiazines as antimicrobials, the upregulation of *ramA* and *acrA* suggest that use of such compounds may also be selective for efflux mutants. However, if this were the case it could also be argued that this point may be irrelevant as such compounds would only be used to treat infections that are already untreatable by other antimicrobials.

This suggests that if the exact nature of the antimicrobial effects and cellular interactions of the phenothiazines could be elucidated, then these mechanisms could be exploited for the development of new antimicrobials.

The presence of active efflux is increasingly thought of as an important resistance mechanism, particularly among members of the Enterobacteriaceae (Webber and Piddock, 2003). RND family efflux pumps, the most abundant family amongst Gram-negative bacteria, have been shown to have a broad substrate range, demonstrating that the activation of a single resistance mechanism can result in clinical levels of resistance to several classes of antibiotics (Webber and Piddock, 2003). It could be argued that efflux is one of the most problematic resistance mechanisms and that ideally any new antibiotics under development should be able to evade such mechanisms. Although only licensed in 2005, tigecycline resistance has quickly arisen in a number of clinically important pathogens including *K. pneumoniae* and *A. baumannii*, largely due to efflux mechanisms (Ruzin *et al*, 2005; Peleg *et al*, 2007). It is possible that tigecycline resistance arises due to its apparent targeting and subsequent upregulation of regulator genes, such as *ramA*, or genes related to the regulation of the regulators as has been observed in this study. Tigecycline is reported to display *in vitro* activity against the *E. coli* strains over-expressing the *tet* transporters (*tet*(B), *tet*(C), *tet*(K)), suggesting that it is capable of evading efflux by the *tet* transporters, an important mechanism of tetracycline resistance in both Gram-positive and Gram-negative bacteria (Hirata *et al*, 2004). This study suggests that although tigecycline may evade the activity of the *tet*

transporters, they may remain viable substrates of other efflux mechanisms, such as the AcrAB pumps.

In conclusion, the two compounds tested in this study appear to be capable of causing the upregulation of the transcriptional activator, *ramA*, and subsequently the efflux gene, *acrA*. It remains unknown whether this is a permanent or transient effect, and whether both compounds exert their effects in the same manner. It could be hypothesised that *ramA*, like other AraC/XylS family regulators, will itself be regulated by a local activator or repressor gene. In this case it could be hypothesised that chlorpromazine or tigecycline acts upon this gene, which in turn upregulates *ramA* and causes a cascade of knock-on effects within the *ramA* regulon, one of which being the upregulation of *acrA*. Alternatively, the compounds may act in another unforeseen manner that warrants further investigation, particularly in the case of tigecycline; an antibiotic currently used as a last resort for the treatment of a number of bacterial infections (Noskins, 2005).

Chapter 4. The role of RamA and RamR in Kp342

4.1 Introduction

4.1.1 Antibiotic resistance in endophyte strain, Kp342

Kp342 is a nitrogen fixing *K. pneumoniae* endophytic strain, typically isolated from maize and wheat crops, the genome sequence of which is available on NCBI (NC 011283) (Fouts *et al*, 2008). The presence of *Klebsiella* spp. in plants can, in part, be attributed to their lack of flagella, a structure that typically induces the plant defence mechanisms in the cases of other bacteria. Endophytic *Klebsiella* spp. have been reported and isolated in a number of different types of plants including sweet potato, rice, maize and bananas (Fouts *et al*, 2008). It is thought that such species can promote the growth of the plant via the provision of fixed nitrogen. Despite being a plant bacterial strain and therefore not exposed to antibiotics, Kp342 has been shown to exhibit high levels of resistance to several families of antibiotics, including the fluoroquinolones and tetracyclines (Fouts *et al*, 2008). Kp342 has been shown to show limited pathogenicity in comparison to clinical isolates in studies using mouse models, indicating that, despite its antibiotic resistance profile, Kp342 is not well adapted for human infection (Fouts *et al*, 2008). In comparison to the sequenced *K. pneumoniae* clinical strain MGH 78578 (NC 009648), Kp342 shows nucleotide and protein identities of 95% and 96% respectively (Fouts *et al*, 2008), indicating a high level of homology despite their contrasting ecological niches. Due to its unusual

resistance profile, Kp342 was chosen for further investigation into the mechanisms responsible.

4.2 Results

4.2.1 Susceptibility testing

Susceptibility testing showed that Kp342 exhibited resistance against norfloxacin (Nor), chloramphenicol (Cm), minocycline (Mino), tetracycline (Tet) and tigecycline (Tige), and intermediate levels of resistance against ciprofloxacin (Cip) (Table 4.1). This indicates that Kp342 exhibits an MDR phenotype.

	MIC (µg/ml)					
	Cip	**Nor**	**Cm**	**Mino**	**Tet**	**Tige**
Range tested	0.0625-512	0.0625-512	0.0625-512	0.0625-32	0.0625-512	0.0625-16
	0.5	4	256	>32	16	4
S/I/R	I	R	R	R	R	R

Table 4.1. **Susceptibility testing results of Kp342 against selected antibiotics.** Resistance break points were determined according to BSAC guidelines. S= sensitive, I = intermediate, R = resistant.

4.2.2 *ramR* sequencing

As *ramA* was previously shown to be over-expressed in Kp342, *ramR* a putative regulator gene that lies upstream of *ramA*, was sequenced and compared to the *ramR* nucleotide sequence of *K. pneumoniae* MGH 78578 (NC 009648). The Kp342 *ramR* nucleotide sequence was shown to contain the following differences as listed in Table 4.2.

Nucleotide Position	MGH 78578	Kp342	Amino Acid Change
78	A	C	None
111	C	T	None
114	T	G	None
126	A	G	None
229	G	C	D77H
312	A	G	None
340-435	N/A	96nt deletion	32aa deletion
456	C	T	None
480	A	G	None
556	C	A	H186N

Table 4.2. ***ramR* sequences.** Differences found in the *ramR* nucleotide sequence of Kp342 compared to the sequence found in MGH 78578 and the resultant amino acid changes. N/A = not applicable.

The translated amino acid sequence of RamR in Kp342 was found to contain a 32 amino acid deletion at position 114-145 as well as two single amino acid changes, D77H and H186N when compared again to that of MGH 78578. Figures 4.1 and 4.2 show the nucleotide and protein sequence alignments.

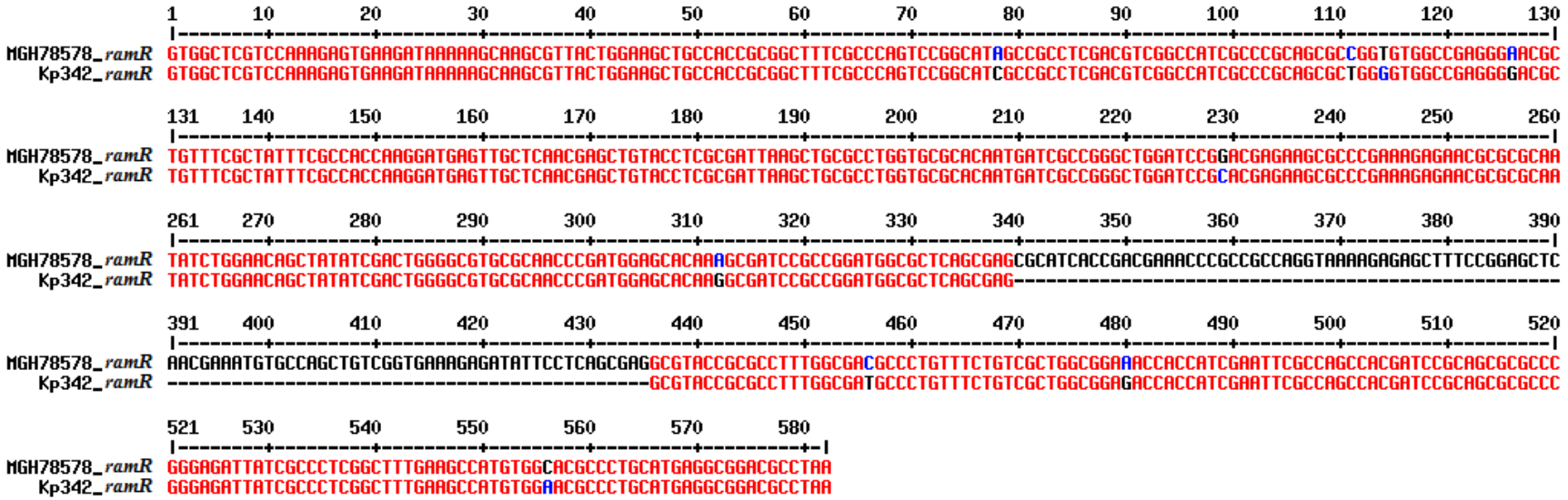


Figure 4.1. **The nucleotide sequence alignments of *ramR* from MGH 78578 and Kp342.**

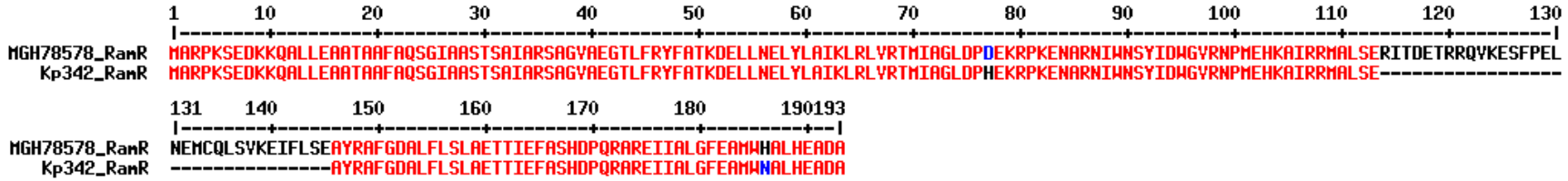


Figure 4.2. **The protein sequence alignments of RamR from MGH 78578 and Kp342.**

4.2.3 *ramR* complementation

Wild-type *ramR,* amplified from MGH 78578, was cloned into the plasmid vector pACYC177, thereafter known as pAC*ramR*, and transformed into Kp342. pACYC177 was also introduced into Kp342 as a vector only control. Kp342, Kp342/pACYC177 and Kp342/pAC*ramR* were subject to susceptibility testing against ciprofloxacin (Cip), norfloxacin (Nor), tetracycline (Tet) and chloramphenicol (Cm). Kp342 and Kp342/pACYC177 were shown to exhibit identical resistance profiles to one another, indicating that the introduction of pACYC177 alone had no effect on the susceptibility phenotype of Kp342, whilst Kp342/pAC*ramR* was shown to exhibit increased susceptibility to all of the antibiotics tested, ranging from 4-16-fold (Table 4.3). The increases in susceptibility suggest that an attenuated or non-functioning RamR may be responsible for the MDR phenotype exhibited by Kp342 and it could be hypothesised that *ramA* and *acrA* are likely to be over-expressed.

	MIC (µg/ml)							
Strain	**Cip**	**Fold Decrease**	**Nor**	**Fold Decrease**	**Tet**	**Fold Decrease**	**Cm**	**Fold Decrease**
Range Tested	0.0625-32		0.0625-32		0.0625-32		0.0625-512	
Kp342	0.5	N/A	4	N/A	16	N/A	256	N/A
Kp342/pACYC177	0.5	0	4	0	16	0	256	0
Kp342/pAC*ramR*	0.125	4	1	4	4	4	16	16

Table 4.3. **Susceptibility testing results for Kp342, Kp342/pACYC177 and Kp342/pAC*ramR*.** N/A = not applicable.

4.2.4 RT-PCR analysis of *ramA* and its associated genes

RT-PCR was performed on RNA extracted from exponential growth cultures of Kp342, Kp342/pACYC177 and Kp342/pAC*ramR* in order to assess the transcription levels of *ramA*, *romA*, *acrA*, and *16S*. *ramA*, *romA* and *acrA* were found to be over-expressed in Kp342 and Kp342/pACYC177 relative to Kp342/pAC*ramR*. Figure 4.3 shows the differences in expression levels of *ramA*, *romA* and *acrA* in Kp342 and Kp342/pACYC177, both of which showed very similar levels of expression, compared to the levels observed in Kp342/pAC*ramR*. All PCRs were performed in duplicate and all calculations were normalised to 16S expression levels. Figure 4.4 shows the agarose gel pictures of the *16S*, *ramA*, *romA* and *acrA* PCRs.

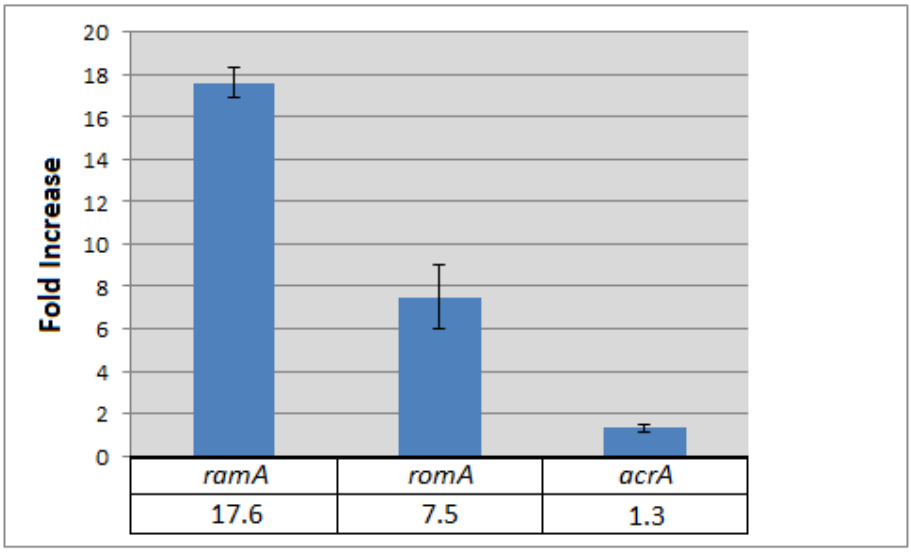


Figure 4.3. **Expression levels of *ramA*, *romA* and *acrA*.** The levels expression of genes *ramA*, *romA* and *acrA* in Kp342 and Kp342/pACYC177 compared to Kp342/pAC*ramR*. Error bars show SEM.

Expression levels were calculated using the GelDoc Quantity One software to measure the intensity of the bands on the agarose gels relative to the 16S internal controls and then compared between strains. Both *ramA* and *romA* did not appear to be expressed in Kp342/pAC*ramR* which translated to a 17.6-fold and 7.5-fold reduction respectively in expression compared to Kp342 and Kp342/pACYC177. *acrA* was deemed to be expressed 1.3-fold more in Kp342 and Kp342/pACYC177 compared to Kp342/pAC*ramR*. Although a certain degree of inaccuracy lies in the GelDoc Quantity One software for the calculation of transcription levels between these strains, the differences in expression levels can be clearly observed in the agarose gel picture in Figure 4.4.

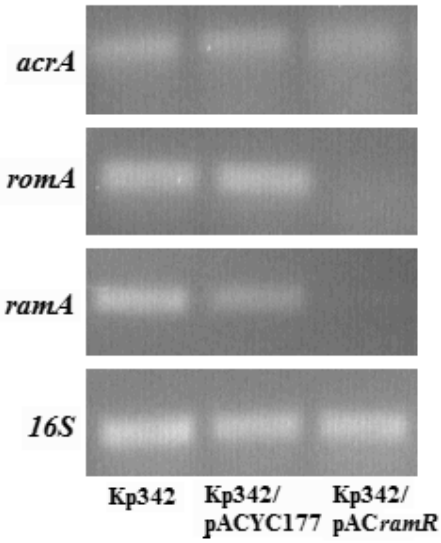


Figure 4.4. **Example gel showing expression of *ramA*, *romA* and *acrA* in Kp342 strains.** PCRs performed in cDNA from Kp342, Kp342/pACYC177 and Kp342/pAC*ramR*.

These results suggest that the RamR present in wild-type Kp342 is non-functional and that RamR acts as a negative regulator of *ramA*, *romA* and *acrA* expression. This suggests that RamA is constitutively expressed in Kp342, resulting in the constant upregulation of the efflux pump, AcrAB.

4.2.5 Biofilm assays

Upon the growth of Kp342, Kp342/pACYC177 and Kp342/pAC*ramR* cultures in LB broth, it was observed that Kp342/pAC*ramR* appeared to form a biofilm around the edges of the glass conical flasks. Biofilm assays were performed on all three strains in order to measure any differences between the levels of biofilm production. Assays were performed in triplicate over 72 hours and the results are shown in Figure 4.5.

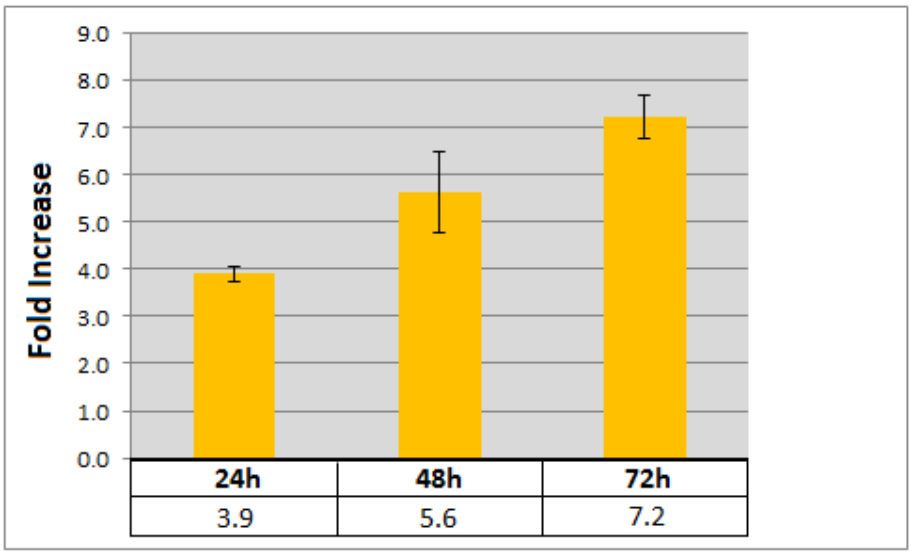


Figure 4.5. **Graph showing the differences in biofilm production between Kp342 strains.** Graph bars show the fold increase in biofilm production between Kp342/pAC*ramR* against averaged Kp342 and Kp342/pACYC177 biofilm measurements over a 72 hour period minus the background readings. Bars show SEM.

An increase in biofilm production was observed in Kp342/pAC*ramR* compared to Kp342 and Kp342/pACYC177 which showed very similar results to each other, indicating that the introduction of pACYC177 alone has no effect on biofilm production in Kp342. The differences were shown to increase over a 72 hour period ranging from a 3.9-fold increase at 24 hours to a 7.2-fold increase at 72 hours.

4.2.6 *romA* cloning

In order to elucidate further the role of *romA,* the gene amplified from MGH 78578, was cloned into vector pACYC177, thereafter known as pAC*romA*. pAC*romA* was transformed into *K. pneumoniae* strain Ecl8 and *E. coli* strain MG1655, as well as pACYC177 only as a vector only control. The strains were subject to susceptibility testing against ciprofloxacin (Cip), chloramphenicol (Cm), tetracycline (Tet) and tigecycline (Tige). The introduction of both *romA* and the vector only control had no effect on the susceptibility profiles of either strain, and so no conclusions could be drawn from this regarding the function of *romA*.

	MIC (µg/ml)			
	Cip	**Cm**	**Tet**	**Tige**
Range Tested	0.008-32	0.0625-128	0.0625-128	0.0625-16
Strain				
Ecl8	0.0156	0.5	0.5	0.125
Ecl8/pACYC177	0.0156	0.5	0.5	0.125
Ecl8/pAC*romA*	0.0156	0.5	0.5	0.125
E. coli MG1655	0.0312	8	1	0.125
E. coli MG1655/pACYC177	0.0312	8	1	0.125
E. coli MG1655/pAC*romA*	0.0312	8	1	0.125

Table 4.4. **Susceptibility testing results of Ecl8 and MG1655 *romA* transformants.**

4.3 Discussion

The Kp342 genome encodes 422 ABC family (7.3% of proteins) and 128 MFS transporters (2.2% of proteins), which are used in the uptake of nutrients, and the extrusion of waste and toxic compounds (Fouts *et al*, 2008). These statistics indicate that Kp342 is likely to encode a number of efflux pumps which may be capable of the efflux of a broad range of substrates. The levels of antibiotic resistance observed in Kp342 may be as a consequence for the bacteria's need to remove toxic plant metabolites, most likely through efflux mechanisms, which may be similar in structure to many antibiotics (Fouts *et al*, 2008). Although Kp342 has been indicated to be an unlikely cause of infection in humans according to results in mouse model studies (Fouts *et al*, 2008), it can be inferred that the mechanisms by which Kp342 confers resistance to antibiotics are likely to be the same as those employed by clinical *K. pneumoniae* strains. These high levels of resistance and the availability of the Kp342 genome sequence (NC 011283) make this strain a good candidate for the study of *K. pneumoniae* antibiotic resistance mechanisms.

The high levels of antibiotic resistance exhibited to the fluoroquinolones, tetracyclines and chloramphenicol in Kp342 can be indicative of a number of mechanisms. *gyrA, gyrB, parC* and *parE* mutations have previously been reported to be responsible for high levels of fluoroquinolone resistance in *E. coli* (Bagel *et al*, 1999), whilst active efflux has been reported to be capable of conferring resistance to all three antibiotic classes (Elkins and Nikaido, 2002). The susceptibility testing results of Kp342 suggest that efflux is likely to be mechanism to be responsible for

conferring such levels of resistance due to the resistance phenotype spanning at least three antibiotic classes, resistance to all of which has previously been associated with an efflux phenotype (Elkins and Nikaido, 2002). The low levels of ciprofloxacin resistance can be attributed to the fact that efflux only has been reported to be inadequate at conferring high levels of resistance in *K. pneumoniae* and the addition of *gyrA* and *parC* mutations are also deemed necessary for high-level resistance (Bratu *et al*, 2009), and Kp342 was shown to contain no mutations within its *gyrA*, *gyrB*, *parC* or *parE* which have been associated with resistance (Fouts *et al*, 2008).

It has previously been reported in *K. pneumoniae* that the transcriptional activator, RamA, plays a role in the regulation of the RND family efflux pump, AcrAB (Schneiders *et al*, 2003). *ramR*, a gene encoding a TetR family protein that lies upstream of *ramA*, has been reported to act as a negative regulator of *ramA* expression in *S. enterica* (Abouzeed *et al*, 2008). The 96 nucleotide deletion identified in the *ramR* gene of Kp342 is highly likely to result in a non-functional or attenuated protein. The subsequent discovery that Kp342 over-expresses *ramA* can therefore be attributed to this defective regulatory gene. The *ramR* complementation studies showed that RamR was responsible for the negative regulation of *ramA* and *romA* expression, which in turn has a regulatory role in the expression of efflux pump gene, *acrA*. The subsequent increases in susceptibility to the fluoroquinolones, tetracyclines and chloramphenicol indicate that the antibiotics tested from these three classes of antibiotic are most likely to be substrates of the AcrAB efflux pump, in agreement with previous literature (Elkins and Nikaido, 2002). Such a drastic deletion of 96 nucleotides in the *ramR* gene of Kp342 could have occurred as a result

of the requirement of plant *K. pneumoniae* strains to incessantly use efflux mechanisms as a means to remove toxic plant metabolites, i.e. to survive in that particular niche, and therefore the function of RamR as a repressor of *ramA* may be obsolete in such strains. Alternatively, endophytic *K. pneumoniae* strains may employ another mechanism by which the expression of *ramA*, *romA* and *acrAB* are regulated, and this mutation is simply the resulting pseudogene. It is however unknown whether the RamR from Kp342 is truly a pseudogene or whether it forms a truncated form of the protein which may or may not continue to serve a function.

The role of RomA remains elusive, although its over-expression alongside *ramA* and repression upon *ramR* complementation, indicate that it is linked to the RamA regulon. A previous study reported that *romA* in *Enterobacter* spp. encodes an outer membrane protein but this remains unconfirmed in *K. pneumoniae* (Komatsu *et al,* 1990), and *romA*'s lack of homology to any other bacterial proteins fails to shed any light on its function. Furthermore the lack of a *romA* gene in the genomes of *Salmonella* spp., where *ramA* and *ramR* are found as in *K. pneumoniae* and *Enterobacter* spp., further complicates the mystery surrounding this gene and its role. The cloning of *romA* in this study did not clarify its role, if any, in antibiotic resistance. Whatever function RomA plays in *K. pneumoniae* and *Enterobacter* spp., it appears to be unimportant for the development of an MDR phenotype in *Salmonella* spp., which can reach levels of antibiotic resistance, comparable to those observed in *K. pneumoniae*, in the absence of a *romA* gene (van der Straaten *et al*, 2004). It is possible that either *Salmonella* spp. have lost the *romA* gene or

alternatively that *K. pneumoniae* and *Enterobacter* spp. have gained this gene as a result of evolutionary pressures.

The content of bacterial genomes are shaped through a combination of events including gene genesis, gene loss, gene gain and horizontal gene transfer. Many bacterial pathogens are thought to have initially evolved through reductive evolution, resulting in smaller, more efficient genomes. Gene loss and gene gain events are likely to work in balance with one another resulting in stable genome sizes as the predominance of either would result in bacterial genomes getting either progressively smaller or bigger. The gene to DNA ration is fairly stable in prokaryotes with roughly 1kb of DNA per gene (Kunin and Ouzounis, 2003). It is therefore quite plausible that RomA may play an important role in *K. pneumoniae* and *Enterobacter* spp. but is not necessary for *Salmonella* spp. to both survive and thrive in its particular ecological niche. For now the exact role of RomA will remain a mystery but it is evident that despite its absence from the *ram* locus in *Salmonella* spp., RomA can be concluded to be linked to RamA in *K. pneumoniae*.

The increase in biofilm production observed upon *ramR* complementation in Kp342 indicate that RamA and RamR may be involved in the regulation of virulence mechanisms, such as biofilm production, in addition to antibiotic resistance. It appears that whilst RamR acts as a repressor of *ramA* expression, it is additionally involved in the activation of biofilm production. The incidence of increased biofilm production upon the increased antibiotic susceptibility following the introduction of the wild-type *ramR* gene, could be indicative of a fitness cost upon the bacterium

when expressing high levels of efflux genes. It has previously been reported that upon *ramA* over-expression and the subsequent *acrAB* over-expression, the major OMP, OmpK35, is down-regulated in *K. pneumoniae,* likely as a compensatory mechanism to maintain the integrity of the outer membrane (George *et al*, 1995). It is possible that the over-expression or repression of *ramA* also affects the expression of other genes associated with the outer membrane such as those involved in the production of biofilm. As Kp342 is an endophytic bacterium, it remains unclear whether this effect is unique to this particular strain, unique to endophytic *K. pneumoniae* strains, or whether it is also observed in clinical strains. It could be worthwhile to investigate whether other endophytic *K. pneumoniae* strains share similar characteristics, particularly the mutations observed in the Kp342 *ramR* gene.

It has been suggested that the over-expression of the AcrAB efflux pump in *K. pneumoniae* can contribute to the virulence of the bacterium (Padilla *et al*, 2010). Padilla *et al* (2010) showed that AcrB deletion mutants exhibited increased susceptibility to antibiotics including the fluoroquinolones, tetracycline, chloramphenicol, aminoglycosides, β-lactams and erythromycin. However lower bacterial loads were also observed in the mutants compared to their wild-type counterparts in mouse lung models, suggesting that AcrAB may play a role in virulence, particularly in the early stages of infection. The authors suggest that AcrAB may be involved in resisting early host defence mechanisms such as antimicrobial peptides. If this were the case then the AcrAB efflux system could play an essential role in the success of *K. pneumoniae*, as well as other members of the Enterobacteriaceae with AcrAB homologues, as a pathogen. This, in turn,

indicates that RamA and RamR may play a role in the regulation of at least one virulence factor via the regulation of this efflux pump.

The results of this study suggest the transcriptional activator RamA plays an important role in the endophytic strain Kp342. The observed deletion in the *ramA* regulator gene *ramR* suggests that the constitutive expression of *ramA* is necessary for the survival of the bacterium in its ecological niche. It would be of additional value to examine the sequences of the *ramR* genes from other *K. pneumoniae* strains in order to identify any common mutations and associated phenotypes to contrast and compare to those found in strain Kp342. It would also be of interest to observe the ability of the Kp342/pACYC*ramR* strain to infect plant tissues compared to its parent Kp342.

Chapter 5: Mutational and functional analysis of the RamR protein

5.1 Introduction

5.1.1 The *ramR* gene

ramR, a gene encoding a TetR-family protein, is encoded upstream and in the opposite orientation of *ramA* and *romA* in *K. pneumoniae* (Figure 5.1).

Figure 5.1. **The genetic arrangement of *ramR*, *romA* and *ramA* in *K. pneumoniae*.**

TetR-family proteins are a large and varied family of proteins, widely dispersed in bacteria and archaea, that typically function as regulatory proteins (Ramos *et al*, 2005). Members of the TetR-family have been shown to play roles in the regulation in a number of essential bacterial processes, ranging from those involved in antibiotic resistance to general cell metabolism (Ramos *et al*, 2005). The role of *ramR* has been speculated, and shown to some extent, to be a local regulator of *ramA* (Abouzeed *et al*, 2008). Abouzeed *et al* (2008) found that the insertional inactivation of *ramR* in *S. enterica* resulted in the subsequent over-expression of *ramA* and reduced susceptibilities to a number of antibiotics, presumably as a result of RamA's role in the regulation of the AcrAB efflux pump. Complementation with an intact

ramR resulted in a reversion to the parental phenotype and so it was concluded that RamR was acting as a repressor of RamA. Its presence, immediately upstream of *ramA*, suggests a role in regulation as the genes encoding the local regulators, MarR and SoxR, of two other AraC/XylS transcriptional activators, are similarly located in close proximity to their regulatory target genes, *marA* and *soxS* (Amabile-Cuevas and Demple, 1991; Alekshun and Levy, 1999). The role and function of RamR was investigated a panel of *K. pneumoniae* strains that included both *ramA* expressers and non-expressers.

5.2 Results

5.2.1 *ramR* sequencing

The sequencing of the entire ORFs of the *ramR* genes from the *K. pneumoniae* panel of strains revealed the following mutations as listed in Table 5.1.

Strain	Nucleotide Position(s) and Changes	Amino Acid Position(s) and Changes	*ramA* Expresser/ Non-Expresser
S7	579: C to A	None	Expresser
S8	No changes	None	Non-Expresser
Ecl8	422: T to C	I141T	Non-Expresser
Ecl8 Mdr1	422: T to C	I141T	Expresser
Kp342	78: A to C, 111: C to T, 114: T to G, 126: A to G, 229: G to C, 312: A to G, Δ340-435, 456: C to T, 480: A to G, 556: C to A	D77H; Δ114-145; H186N	Expresser
S28	Δ440-453, 496: G to C, 513: G to A, 514: C to T, 515: G to T, 523: G to C, 547: G to A, 572: C to T	F/S and stop codon at 147	Expresser
S29	Insertion 488; ACCACCA	F/S from 163; stop codon at 183	Expresser
CG43	No changes	None	Non-Expresser

Table 5.1. **The results of the sequencing of *ramR* from the panel of *K. pneumoniae* strains.** The changes observed in the nucleotide gene sequence and the resultant amino acid changes for each strain are listed. The *ramA* expression status is also included. Changes are noted in regard to the *ramR* gene sequence from MGH 78578 (YP_001334235). F/S = frame-shift.

All of the *ramA* over-expressing strains contained deletions or insertions that either caused frame-shifts or were likely to affect the RamA protein structure, with the exception of Ecl8 Mdr1 and S7, both of which had protein sequences identical to that of the *ramA* non-expressing strain MGH 78578. Ecl8 and Ecl8 Mdr1 had identical sequences to one another despite Ecl8 Mdr1 being a *ramA* over-expresser and the strains being isogenic; Ecl8 Mdr1 was derived from Ecl8 via selection with chloramphenicol (George *et al*, 1995). The change from isoleucine to threonine observed in Ecl8 and Ecl8 Mdr1 is unlikely to bear any affect on the function of RamR and *ramA* expression since it is found in both of the strains. Kp342 was shown to contain two amino acid changes; aspartic acid to histidine at position 77 and histidine to asparagine at position 186, additionally a 32 amino acid deletion from the middle of the gene toward the C-terminus, although the gene sequence remains in frame following the deletion. Such a change is likely to have a significant effect on the structure of the RamR protein and subsequently its ability to function. Strain S28 contained a number of silent changes at the nucleotide level, however the most significant change was a 14nt deletion which resulted in a premature stop codon at amino acid position 147, resulting in a truncated RamR protein. Strain S29 contained a 7nt insertion, which resulted in a frame-shift from amino acid position 163 and a premature stop codon at 183, causing a truncated RamR protein. As expected, no major changes were observed in the *ramR* gene sequences of the *ramA* non-expressing strains. Alignments of the RamR protein sequences are shown in Figure 5.2.

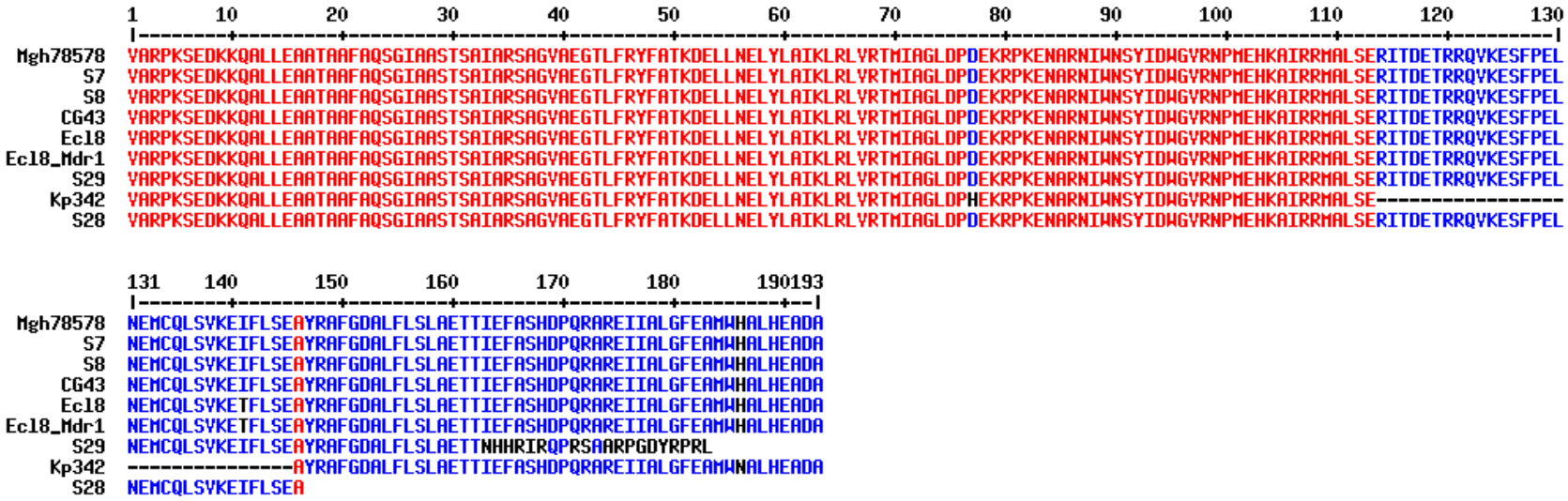


Figure 5.2. **The protein sequence alignments of RamR from the *K. pneumoniae* panel of strains.**

5.2.2 *ramR* mutation studies

CPZ, ciprofloxacin and tigecycline mutants of Ecl8 were obtained by culturing on media containing 8X the respective MICs of each compound. Two mutants were selected from the ciprofloxacin and tigecycline plates and one from the chlorpromazine plate, and subjected to susceptibility testing against chloramphenicol (Cm), ciprofloxacin (Cip), norfloxacin (Nor) and tetracycline (Tet). All of the mutants exhibited significantly higher MICs against the tested antibiotics compared to Ecl8 (Table 5.2). The increases in MICs ranged from 8-fold to 128-fold.

	MIC (µg/ml)							
	Cip	**Fold Increase**	**Nor**	**Fold Increase**	**Tet**	**Fold Increase**	**Cm**	**Fold Increase**
Range Tested	0.0156-256		0.0156-256		0.0156-256		0.0156-256	
Strain								
Ecl8	0.0156	N/A	0.0625	N/A	0.5	N/A	0.5	N/A
Ecl8-CPZ-1	0.25	16	1	16	8	16	16	32
Ecl8-Tig-1	0.25	16	1	16	8	16	16	32
Ecl8-Tig-2	0.25	16	1	16	8	16	16	32
Ecl8-Cip-1	0.5	32	4	64	4	8	64	128
Ecl8-Cip-2	0.25	16	2	32	8	16	32	64

Table 5.2. **Susceptibility testing results of Ecl8 and the Ecl8 mutants.**

NA = not applicable.

5.2.3 Mutant *ramR* sequencing

The *ramR* gene from the Ecl8 mutants was subsequently amplified and sequenced. The following mutations were found (Table 5.3). Nucleotide mutations resulting in

changes at the amino acid level were found in all mutants with the exception of Ecl8-Cip-1 which had a *ramR* sequence identical to that of Ecl8. Ecl8-Cip-2 contained two nucleotide mutations, which resulted in two amino acid changes; glutamic acid to lysine at position 175 and aspartic acid to asparagine at position 192. Ecl8-CPZ-1 contained, potentially the most significant change, resulting in a premature stop codon at position 137. The two tigecycline mutants, Tig-1 and Tig-2, contained the same single nucleotide and amino acid change; glycine to aspartic acid at position 96. All of the observed mutations could potentially hinder the function of RamR as a repressor of *ramA*.

Strain	Nucleotide Position(s) and Changes	Amino Acid Position(s) and Changes
Ecl8-CPZ-1	410: C to A	S137stop
Ecl8-Cip-1	No changes	No changes
Ecl8-Cip-2	523: G to A; 574: G to A	E175K; D192N
Ecl8-Tig-1	287: G to A	G96D
Ecl8-Tig-2	287: G to A	G96D

Table 5.3. **The nucleotide changes found in Ecl8 mutants and their resultant amino acid changes.**

Figure 5.3 shows the protein sequence alignments of the Ecl8 mutants vs Ecl8 itself. Although no particular mutation hot spots can be observed in the RamR sequences from these few mutants, it does appear that mutations appear to be directed towards the C-terminus rather than the N-terminus.

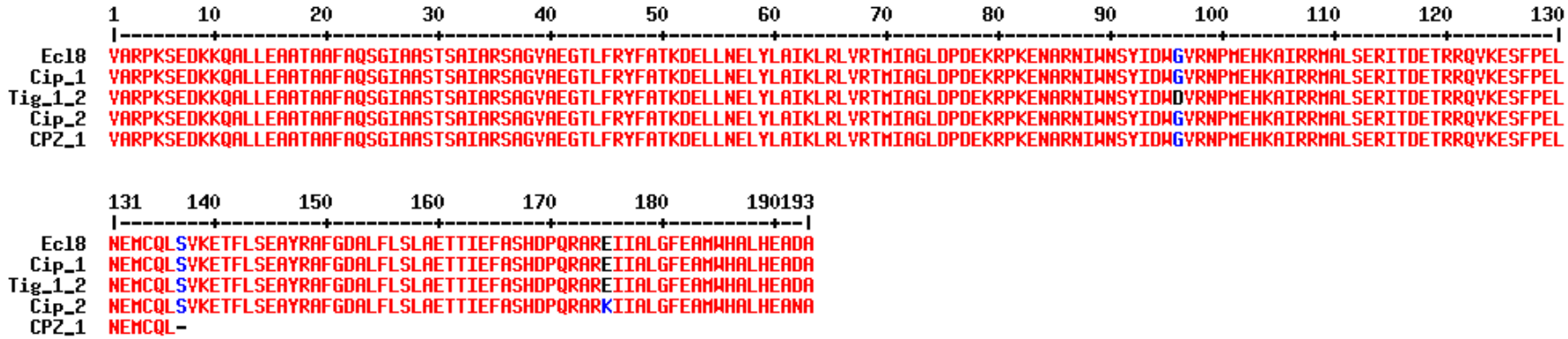


Figure 5.3. **The protein sequence alignments of RamR from Ecl8 and the Ecl8 mutants.**

5.2.4 *ramR* complementation

pAC*ramR* and pACYC177 were transformed into Ecl8, Ecl8 Mdr1 and each of the Ecl8 mutants. The introduction of pACYC177 and pAC*ramR* into Ecl8 and Ecl8 Mdr1 had no effect on their susceptibility profiles to the antibiotics tested (Table 5.4). This is indicative that additional *ramR* expression in strains already expressing a functional RamR has no effect on susceptibility profiles, signifying that the susceptibility profiles displayed by Ecl8 Mdr1 are not attributable to a non-functioning or attenuated RamR protein. Additionally, this suggests that the effects of RamR are not subject to a dose dependent effect in these strains. It is clear from the data in Table 5.4 that the pACYC177, vector only control, similarly had no effect on the susceptibility profile of the mutants with the exception of Cip-2 where a small increase is susceptibility was observed to both norfloxacin and chloramphenicol, likely to be an insignificant effect. The introduction of pAC*ramR* into Ecl8-CPZ-1 increased the susceptibility of the strain to ciprofloxacin (2-fold), tetracycline (2-fold) and chloramphenicol (4-fold), however no change was observed in the MIC of norfloxacin. Ecl8-Tig-1 and Ecl8-Tig-2, unsurprisingly since they contained the same amino acid change, showed the same antibiotic profiles following transformation with pAC*ramR*. Susceptibility was increased to ciprofloxacin (16-fold), norfloxacin (8-fold), tetracycline (8-fold) and chloramphenicol (4-fold). Of note, pAC*ramR* resulted in restoration of the wild-type, Ecl8, susceptibility profile in the cases of both ciprofloxacin and tetracycline. The introduction of pAC*ramR* into Ecl8-Cip-1 showed little effect with the exception of a 2-fold increase in susceptibility to tetracycline, which incidentally was also observed in the vector only

control strain. Overall pAC*ramR* appears to have a negligible effect in this strain which is not entirely unexpected as no mutations were identified within its *ramR*. Ecl8-Cip-2 exhibited an increase in susceptibility to ciprofloxacin (2-fold), norfloxacin (2-4-fold, compared to both Ecl8-Cip-2P and Ecl8-Cip-2R), tetracycline (8-fold) and chloramphenicol (4-8-fold, compared to both Ecl8-Cip-2P and Ecl8-Cip-2R). Of note, the tetracycline MIC of Ecl8-Cip-2R was restored to the same as that of Ecl8.

	MIC (μg/ml)							
	Cip		Nor		Cm		Tet	
Range Tested	0.0625-512		0.0625-512		0.0625-512		0.0625-16	
Strain		**Fold Change From Ecl8**		**Fold Change From Ecl8**		**Fold Change From Ecl8**		**Fold Change From Ecl8**
Ecl8	0.0156	**N/A**	0.0625	**N/A**	0.5	**N/A**	0.5	**N/A**
Ecl8-P	0.0156	**0**	0.0625	**0**	0.5	**0**	0.5	**0**
Ecl8-R	0.0156	**0**	0.0625	**0**	0.5	**0**	0.5	**0**
Ecl8 Mdr1	8	**N/A**	128	**N/A**	128	**N/A**	256	**N/A**
Ecl8 Mdr1-P	8	**N/A**	128	**N/A**	128	**N/A**	256	**N/A**
Ecl8 Mdr1-R	8	**N/A**	128	**N/A**	128	**N/A**	256	**N/A**
Ecl8-CPZ-1	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-CPZ-1P	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-CPZ-1R	0.125	**8**	1	**16**	4	**8**	2	**4**
Ecl8-Tig-1	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-Tig-1P	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-Tig-1R	0.0156	**0**	0.125	**2**	4	**8**	0.5	**0**
Ecl8-Tig-2	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-Tig-2P	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-Tig-2R	0.0156	**0**	0.125	**2**	4	**8**	0.5	**0**
Ecl8-Cip-1	0.5	**32**	4	**64**	64	**128**	2	**4**
Ecl8-Cip-1P	0.5	**32**	4	**64**	64	**128**	1	**2**
Ecl8-Cip-1R	0.5	**32**	4	**64**	64	**128**	1	**2**
Ecl8-Cip-2	0.25	**16**	2	**32**	32	**64**	4	**8**
Ecl8-Cip-2P	0.25	**16**	1	**16**	16	**32**	4	**8**
Ecl8-Cip-2R	0.125	**8**	0.5	**8**	4	**8**	0.5	**0**

Table 5.4. **Susceptibility testing results of Ecl8 and the Ecl8 mutants after transformation with pACYC177 and pAC*ramR*.** Fold changes in MIC are shown in respect to Ecl8's susceptibility profile. N/A = not applicable.

5.3 Discussion

The results of this study clearly indicate that RamR plays an important role in the regulation of *ramA* expression, however they also suggest that RamR is not the sole regulator of the *ram* regulon, but rather is acting as a local repressor.

The analysis of the panel of *K. pneumoniae* strains showed that whilst in some strains *ramA* over-expression could be attributed to mutations within the *ramR* gene, in other strains, such as Ecl8 Mdr1 and S7, another regulatory mechanism may be responsible. In the cases of strains Kp342, S28 and S29, there are significant mutations that are likely to result in either truncated proteins or changes in protein conformation that would likely hinder or completely disable the function of the RamR protein. The two exceptions to this finding are the *ramR* sequences of Ecl8 Mdr1 and S7, both of which are *ramA* over-expressers that had no significant changes within their *ramR* gene sequences. The lack of explanation within the *ramR* gene for the over-expression of *ramA* in these two strains can be due to a number of reasons, for example, another gene may also regulate the expression of *ramA* or alternatively, *ramR* - which in turn affects *ramA*. The capacity of the *ramA* regulon remains largely unexplored and it is unknown how far its reach extends and the factors involved in its regulation. Another two members of the AraC/XylS family of transcriptional regulators, MarA and SoxS, were shown by microarray analysis to upregulate 153 genes upon their induction by chemicals *in vitro*, although it is unknown how many of these genes are directly regulated by MarA and SoxS *in vivo* (Martin and Rosner, 2002). Although RamR appears to act as a local repressor of

ramA, it is also reasonable to expect that the expression of *ramA* will additionally be subject to regulation by other, as yet unknown, mechanisms.

The mutational studies showed that mutations within the *ramR* gene were reasonably easy to obtain by the use of chlorpromazine, ciprofloxacin and tigecycline. It appears as though these mutations were likely to be targeted towards the *ramR* gene as all three chemicals are plausible substrates of the AcrAB efflux pumps that are regulated by RamA. The mutation found within the CPZ-1 mutant resulting in a premature stop codon and therefore a truncated protein, would be predicted to have the most prominent effect on *ramA* and subsequently the MICs. In all of the mutated *ramR* strains, the MICs to the tested antibiotics increased significantly (8-128-fold) resulting in intermediate or resistant levels for all antibiotics except ciprofloxacin, which despite a significant decrease in susceptibility the mutants remained clinically sensitive. This is not surprising as Bratu *et al* (2009) reported that fluoroquinolone resistance in *K. pneumoniae* was less commonly mediated through RamA and AcrAB but instead was more likely to be as a result of *gyrA* and *parC* mutations. However, it was observed that the Cip-1 mutant exhibited the greatest fold changes in MICs despite containing no mutation within its *ramR* gene. This result again indicates that the conferring of an MDR phenotype and the regulation of *ramA* may be controlled by another regulator distinct from RamR. The increases in MICs observed in all of the mutants with changes within *ramR* are consistent with the phenotype associated with *ramA* and subsequent *acrAB* over-expression.

The complementation studies showed that the presence of the vector only, pACYC177, or the *ramR* complement, pACYC*ramR*, had no effect on the MICs of the parent strain Ecl8. This indicates that the excess of RamR produced by the plasmid vector does not further increase susceptibility to antibiotics. This is likely to be due to the fact that the *ramA* gene is not expressed in Ecl8 and therefore the *ramR* expressed from the plasmid has no further role in regulation in this strain. Additionally the introduction of pACYC177 and pAC*ramR* into Ecl8 Mdr1 did not affect MICs indicating that any malfunction in the RamR protein is not responsible for *ramA* over-expression in Ecl8 Mdr1 and so another mechanism is responsible in this case. The complementation of *ramR* into the Ecl8 mutants showed varying degrees of increased susceptibility and, in some cases, returning the mutants to its parental phenotype for that particular antibiotic. This indicates that the mutations within *ramR* have a significant effect on the function of the protein, most likely hindering the repression of *ramA*. The lack of restoration of MICs in the Cip-1 mutant upon *ramR* complementation was to be expected, as this was the only mutant that did not have any mutations within the *ramR* gene and subsequently the MDR phenotype must be caused by an alternative mechanism. Although *ramR* complementation resulted in the restoration of the parental phenotype in some of the mutants (Tig-1 and Tig-2 showed complete restoration of their MICs to ciprofloxacin and tetracycline, and Cip-2 also showed complete restoration of its MIC to tetracycline), for most there was just partial restoration. This may indicate that the chemicals used to obtain the mutants are also responsible for causing other mutations in addition to those observed in *ramR*, that contribute to the antibiotic resistance phenotype.

Figure 5.4 illustrates the alignment of the RamR protein sequence from *K. pneumoniae* MGH 78578, *Enterobacter aerogenes* 638 and *S. typhimurium* LT2. Highlighted in the figure are mutations in RamR found in this study as well as those found in other studies (Abouzeed *et al*, 2008; Molitor, 2008; Hentschke *et al*, 2010). The figure illustrates that the majority of mutations are located towards the C-terminus rather than around the DNA binding domain or HTHs. As might be expected, the DNA binding domain and HTH motifs are well conserved in the RamR sequences of all three species, although it should also be noted that the levels of conservation between species outside of these domains is significantly lower. Whilst some of the mutations may not affect the action of the RamR protein in its role as a regulator of RamA, particularly the mutations that occurred in residues that are not conserved between species, there does not seem to be one particular hotspot or residue that is commonly mutated. There does, however, appear to be a higher incidence of mutations directed toward the C-terminus.

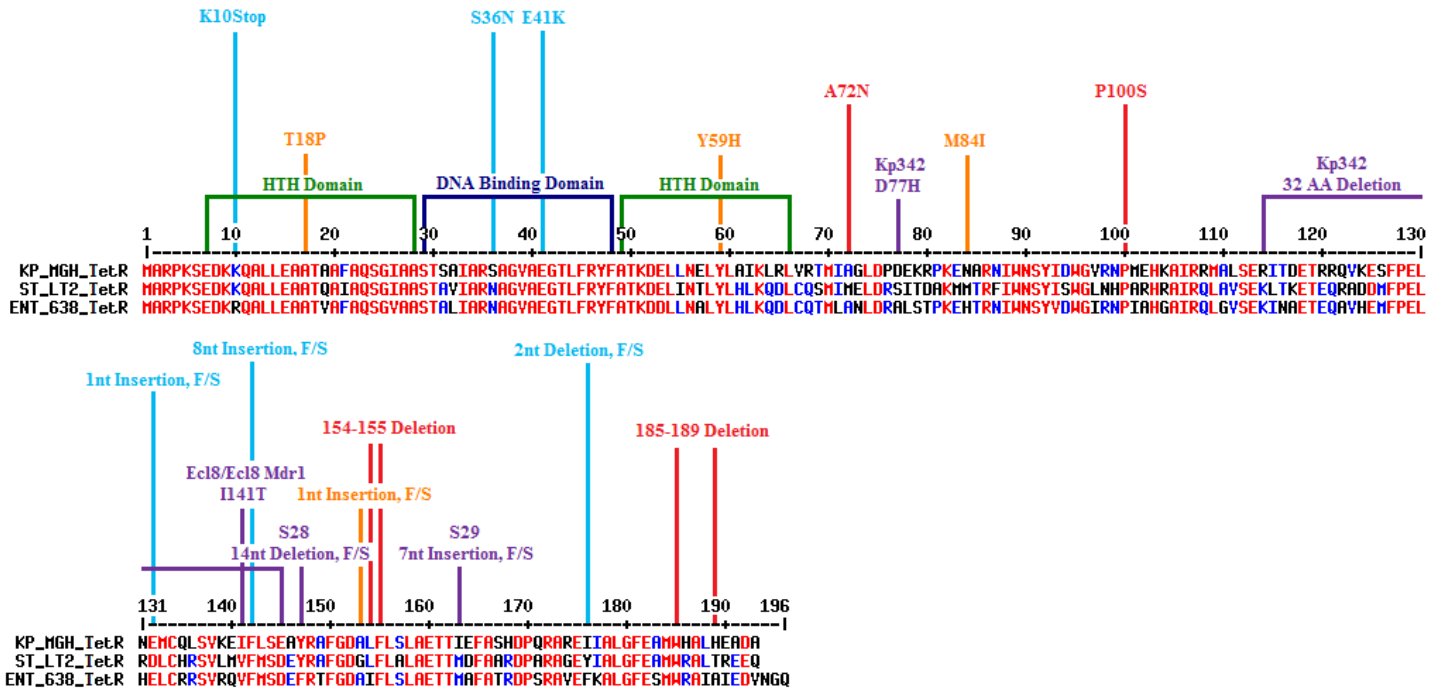


Figure 5.4. **An alignment of the protein sequences of RamR from *K. pneumoniae* MGH 78578, *S. enterica* Typhimurium LT2 and *Enterobacter* 638.** The changes observed in the strains in this study are denoted in purple. The changes in orange were observed by Abouzeed *et al* (2008) in *S. typhimurium*; changes in red were observed by Molitor (2008) in *E. aerogenes*; changes in blue were observed by Hentschke *et al* (2010) in *K. pneumoniae*. DNA binding domains and HTH domains are as indicated by Molitor (2008).

The results of this study clearly indicate that the regulation of RamA is a complex process. Whilst it has been shown that RamR is responsible for RamA regulation in some strains of *K. pneumoniae*, such as Kp342, it has also been shown that in other strains, Ecl8, Ecl8 Mdr1 and S7, that RamR plays no obvious role. Mutations within RamR are clearly associated with *ramA* over-expression in the panel of *K. pneumoniae* strains in this study, however it remains unclear which other factors are involved in conferring this effect. It could be speculated that the ability of the chemicals CPZ, ciprofloxacin and tigecycline, to select for mutations within the *ramR* gene appears to be a targeted effect. It should also be noted that the chemicals used for mutant selection are all plausible substrates for the AcrAB efflux pump, which is regulated by RamA, and so mutations that cause its over-expression could be interpreted as a selected and necessary survival mechanism employed by the bacterium.

Further studies are necessary to fully understand the role of RamR and its regulation of RamA. Whilst it is apparent that RamR is not the sole regulator of RamA, it is unknown whether RamR is also a regulator of any other genes. It would be of particular value to perform microarray analyses of strains of *K. pneumoniae* that are both expressers and non-expressers of *ramA*, and additionally those that contain wild-type and mutated *ramR* genes.

Chapter 6: The development of carbapenem resistance

6.1 Introduction

6.1.1 Clinical significance of *K. pneumoniae*

K. pneumoniae HAI's are common within hospitals in the USA and Europe, comprising around 6% of infections according to CDC data (Hidron *et al*, 2008). Although *K. pneumoniae* can cause infection at various sites within the body, it is most commonly isolated from UTIs, often as a result of patient catheterisation (Nicolle, 2008). It is common practice to treat such infections with fluoroquinolones, aminoglycosides or third generation cephalosporins, which are usually adequate to combat the infection within reasonable time limits. However, the increasing incidence of MDR *K. pneumoniae* strains often renders these classes of antibiotics inadequate. The carbapenems are usually reserved for treating *K. pneumoniae* infections that are unresponsive to the aforementioned antibiotics or for more complicated cases, such as blood stream infections or patients with serious underlying medical conditions and are, as such, termed the 'drugs of last resort'. *K. pneumoniae* infections that do not respond to carbapenem therapy present a serious problem and may require alternative antibiotic therapies.

6.1.2 Strain background

A 71 year old male was admitted to Hairmyres Hospital (East Kilbride, Lanarkshire) in May of 2008 with an undisclosed medical condition. The patient was catheterised throughout his stay and developed a UTI soon after his admittance. He was initially treated with ceftriaxone for which the UTI failed to respond. Following this, strain 1, known as K1, was collected on the 8th of May from the patients urine and was subsequently identified as *K. pneumoniae*. The patient was then subjected to meropenem therapy, which also failed to clear the infection. Following the meropenem therapy failure, the patient was then treated with fosfomycin to which he initially appeared to respond but the UTI recurred soon afterward. Strain 2, known as K2, was subsequently collected on the 13th of May, from the urine and again identified as *K. pneumoniae*. Subsequent meropenem treatment also failed and by this point the patient had also developed *Clostridium difficile* and MRSA infections, all of which contributed to his eventual death in the July. Figure 6.1 shows a timeline of the patient's treatment. K2 was sent to the HPA (Colindale, London) where it underwent susceptibility testing and was shown to exhibit resistance to all drugs tested with the exception of colistin. The HPA reported no carbapenemase detection by cloverleaf assay; the mechanism of carbapenem resistance was therefore unknown.

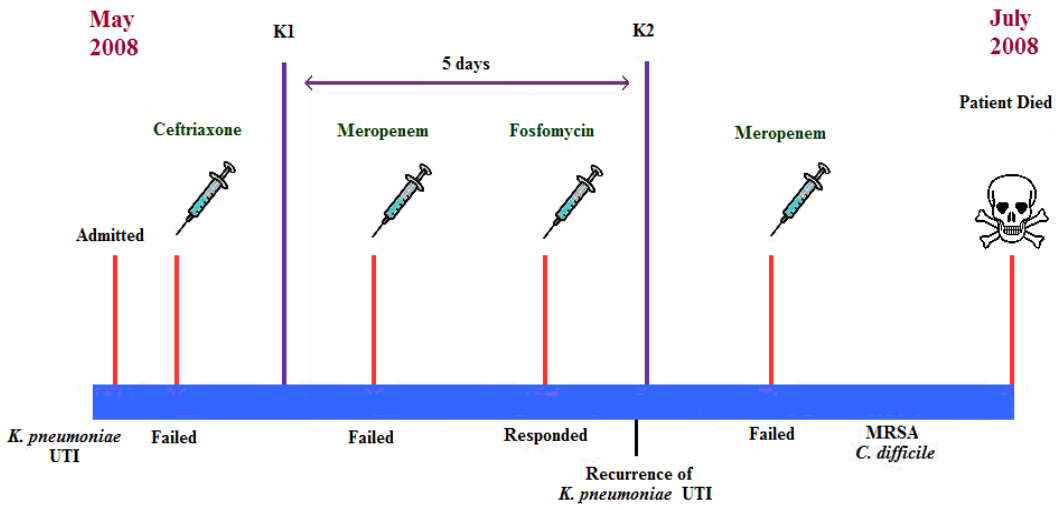


Figure 6.1. **A timeline showing the treatment regimen and response of the patient.** Note: timeline is not to scale.

6.2 Results

6.2.1 Strain typing by PFGE

Strains K1 and K2 were subject to digestion by *Xba*I and PFGE. Both strains gave indistinguishable banding patterns, indicating that the strains are of the same clonal type (Figure 6.2).

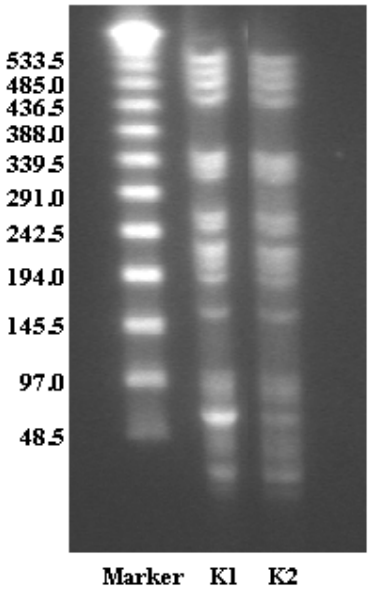


Figure 6.2. **The PFGE gel of K1 and K2.** Strains were digested with *Xba*I. Marker sizes are in kb.

6.2.2 Growth curve

Growth curves performed on both K1 and K2 in LB broth identified that K1 appears to initially grow significantly faster than K2, particularly in the first two hours, but both strains reached a similar OD_{600} as they approached the stationary growth phase (Figure 6.3). K1 exhibited 79%, 47%, 15%, 8%, 5% and 4% increases in OD_{600}

readings over the 6 hours compared to strain K2. This could be indicative of increased fitness in K1 compared to K2.

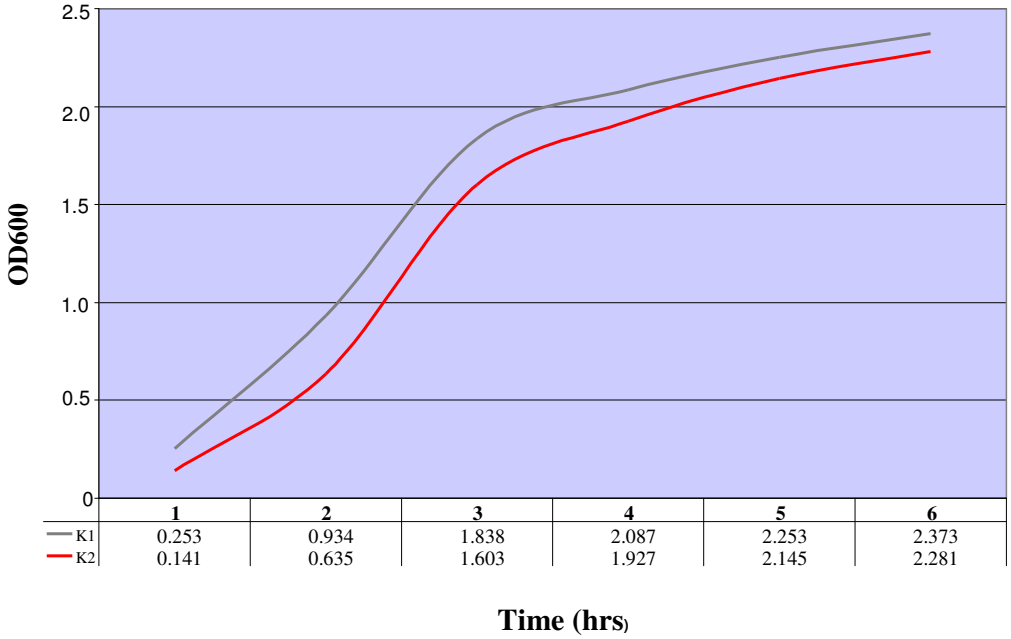


Figure 6.3. **A growth curve of K1 and K2.** Strains were grown in LB broth over 6 hours.

6.2.3 Biofilm assays

It was observed when routinely growing overnight cultures of K1 and K2 in liquid media that K2 appeared to produce more biofilm than K1. Biofilm production was measured comparatively between K1 and K2. Strains were inoculated in LB broth and incubated for 24, 48 and 72 hours respectively. As shown in Figure 6.4, K2 produced more biofilm than K1 over the entire 72 hour time period. K2 exhibited 81%, 55% and 65% increases in biofilm production compared to K1. The differences in the levels of biofilm production between the two strains may not be

substantial enough to be considered clinically significant, but the differences do show a characterised difference between the two strains.

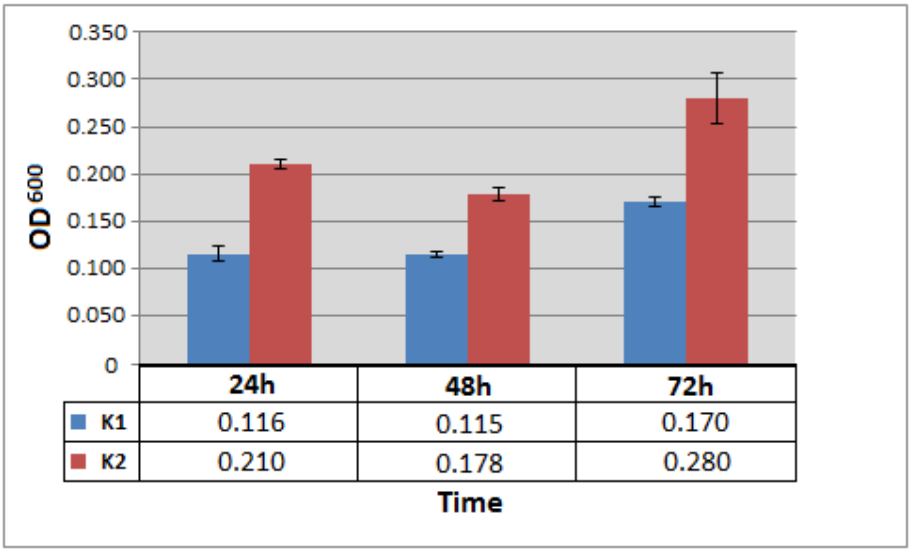


Figure 6.4. **Graph showing the differences in biofilm production between strains K1 and K2.** A comparison of biofilm production in K1 and K2 when grown in LB broth over 72 hours. Error bars show SEM.

6.2.4 Susceptibility testing of K1 and K2

Susceptibility testing was performed by the agar doubling dilution method on K1 and K2 against 17 antibiotics as well as an imipenem/EDTA combination, the results of which are shown in Table 6.1. There was one notable difference between K1 and K2; K2 was resistant to meropenem and ertapenem and had an intermediate MIC to imipenem, whilst K1 was sensitive to all three carbapenems. Aside from this difference, both strains exhibited resistance to all other antibiotics tested with the only other exception being sensitivity to colistin in both strains. Additionally, whilst both strains were resistant to aztreonam and ciprofloxacin, K1 exhibited a

significantly higher MIC to both antibiotics (at least 4-fold higher in both cases) and a lower MIC than K2 to cefoxitin (at least 2-fold). The lack of reduction in MICs between imipenem and the imipenem/EDTA combination indicate that there was unlikely to be a metallo-β-lactamase present in either strain, however the broad spectrum antibiotic resistance exhibited by both strains was indicative of a number of resistance mechanisms.

		MIC (µg/ml)			
Antibiotic	**Range Tested**	**K1**	**S/I/R**	**K2**	**S/I/R**
Amikacin	0.0625-512	32	R	32	R
Ampicillin	0.0625-512	>512	R	>512	R
Aztreonam	0.0625-256	>256	R	64	R
Cefoxitin	0.0625-512	64	R	>128	R
Ceftazidime	0.0625-128	>128	R	>128	R
Chloramphenicol	0.0625-512	64	R	64	R
Ciprofloxacin	0.0625-256	>256	R	64	R
Colistin	0.0625-32	1	S	1	S
Ertapenem	0.0625-32	1	S	64	R
Gentamicin	0.0625-512	128	R	128	R
Imipenem	0.0625-32	0.125	S	4	I
Imipenem/EDTA	0.0625-32	0.125	S	4	I
Meropenem	0.0625-32	0.125	S	8	R
Piperacillin	0.0625-512	>512	R	>512	R
Sulbactam	0.0625-32	>32	R	>32	R
Tetracycline	0.0625-512	16	R	8	R
Tigecycline	0.0625-16	4	R	2	I
Tobramycin	0.0625-512	64	R	64	R

Table 6.1. **Susceptibility testing of strains K1 and K2 against a broad range of antibiotics.** Strains are indicated as being sensitive (S), intermediate (I) or resistant (R) according to BSAC guidelines (BSAC, 2011). *EDTA used at 320µg/ml.

6.2.5 Susceptibility testing in the presence of clavulanic acid

To determine the contribution of clavulanic acid sensitive β-lactamases to the carbapenem resistant phenotype, susceptibility testing was performed in the presence of clavulanic acid for the carbapenems, ertapenem and imipenem, and selected cephalosporins: cefotaxime, cefpodoxime and ceftazidime. Clavulanic acid was incorporated into IST agar at 4µg/ml and discs were used for the antibiotics. A number of strains in addition to K1 and K2 were also included were included to act as controls and as comparators. Results are shown in Table 6.2.

	Zone Diameters (mm)				
	Cefotaxime (30µg)	**Cefpodoxime (10µg)**	**Ceftazidime (30µg)**	**Ertapenem (10µg)**	**Imipenem (10µg)**
K1	0	0	0	26	34
K1/Clav	18	20	0	31	38
Difference	**18**	**20**	**0**	**5**	**4**
K2	0	0	0	0	26
K2/Clav	0	0	0	12	28
Difference	**0**	**0**	**0**	**12**	**2**
MGH 78578 (TEM-1, SHV-12)	35	35	0	40	40
MGH 78578 (TEM-1, SHV-12)/Clav	35	35	0	40	40
Difference	**0**	**0**	**0**	**0**	**0**
EC MG1655	ND	27	ND	37	34
EC MG1655/Clav	ND	27	ND	38	36
Difference	ND	**0**	ND	**1**	**2**
EC ATCC 25922	39	27	34	40	37
EC ATCC 25922/Clav	40	27	35	39	37
Difference	**1**	**0**	**1**	**-1**	**0**
EC TEM-1	42	39	9	45	38
EC TEM-1/Clav	47	40	15	48	44
Difference	**5**	**1**	**6**	**3**	**4**
EC SHV-1	18	8	0	43	47
EC SHV-1/Clav	36	34	0	46	52
Difference	**8**	**26**	**0**	**3**	**4**
KP MB-CTX-M-15	0	0	0	30	30
KP MB-CTX-M-15/Clav	31	29	0	37	35
Difference	**31**	**29**	**0**	**7**	**5**
KP 32 CTX-M-15	0	0	0	28	33
KP 32 CTX-M-15/Clav	25	25	8	36	36
Difference	**25**	**25**	**8**	**8**	**3**
ATCC 13883	ND	ND	ND	38	30
ATCC 13883/Clav	ND	ND	ND	38	30
Difference	ND	ND	ND	**0**	**0**

Table 6.2. **The results of disc susceptibility testing to antibiotics with and without clavulanic acid.** Additional strains: EC and KP strains containing specific β-lactamases (as indicated in strain names) and EC 25922, EC MG1655 – non-β-lactamase containing control strains. Difference values are highlighted in red and those ≥5 are underlined. ND= not determined.

Differences in zone diameters ≥5mm were considered to be significant for the presence of clavulanic sensitive β-lactamases involved in the hydrolysis of that particular antibiotic. The differences in disc diameter for ertapenem in K1, K2, KP MB-CTX-M-15 and KP 32-CTX-M-15 suggested that CTX-M-15 may be a contributing factor in the conferring of ertapenem resistance, showing diameter increases of 5mm, 12mm, 7mm and 8mm respectively. Notably, no or negligible differences were observed in the non-β-lactamase containing control strains MG1655, ATCC 13883 and ATCC 25922 in the presence of clavulanic acid. Additionally MGH 78578, which according to its published genome sequence (NC_009648), contained the β-lactamases TEM-1 and SHV-12, showed no differences in zone diameters. As expected the *E. coli* strains containing the TEM-1 and SHV-1 β-lactamases exhibited increased susceptibility to the cephalosporin antibiotics when tested in the presence of clavulanic acid but no significant differences were observed for either ertapenem or imipenem.

6.2.6 Stability of resistance phenotype in K1 and K2

To assess the stability of the resistance phenotype exhibited by both K1 and K2, both strains were subcultured on nutrient agar for 10 days before subsequent susceptibility testing. It was shown that the subcultured strains (K1S and K2S) exhibited an identical resistance phenotype to their parental counterparts, K1 and K2, with one exception; K2 showed altered susceptibilities to the aminoglycoside antibiotics amikacin, gentamicin and tobramycin (Table 6.3). These alterations in susceptibility to the aminoglycosides result in K2 being rendered sensitive. This may indicate the

loss of an aminoglycoside resistance determinant in the absence of antibiotic pressure.

	MIC (µg/ml)		
Strain	**Amikacin**	**Gentamicin**	**Tobramycin**
K1	32	>128	128
K2	32	>128	128
K1S	32	>128	128
K2S	1	0.25	4

Table 6.3. **Susceptibility testing results of K1, K2 and their subcultured counterparts, K1S and K2S, against the aminoglycoside antibiotics amikacin, gentamicin and tobramycin.**

6.2.7 K2 disc synergy testing

Synergy disc tests were set up for K2 against ertapenem, imipenem and meropenem with amoxicillin/clavulanic acid. Figure 6.5 part 1 shows synergy between imipenem and amoxicillin/clavulanic acid, and part 2 shows synergy between meropenem and amoxicillin/clavulanic acid. These results appear to indicate that there is an enzyme present in K2, capable of the hydrolysis of both imipenem and meropenem that can be inhibited by clavulanic acid. No synergy was observed with ertapenem. Of note the observed synergy effects were subject to optimal spacing of the discs; 25mm in part 1 and 10mm in part 2. The synergy effect observed for imipenem contrasts that of the observations in K2 where no significant difference was shown when using clavulanic incorporated into the agar with imipenem discs (Table 6.2).

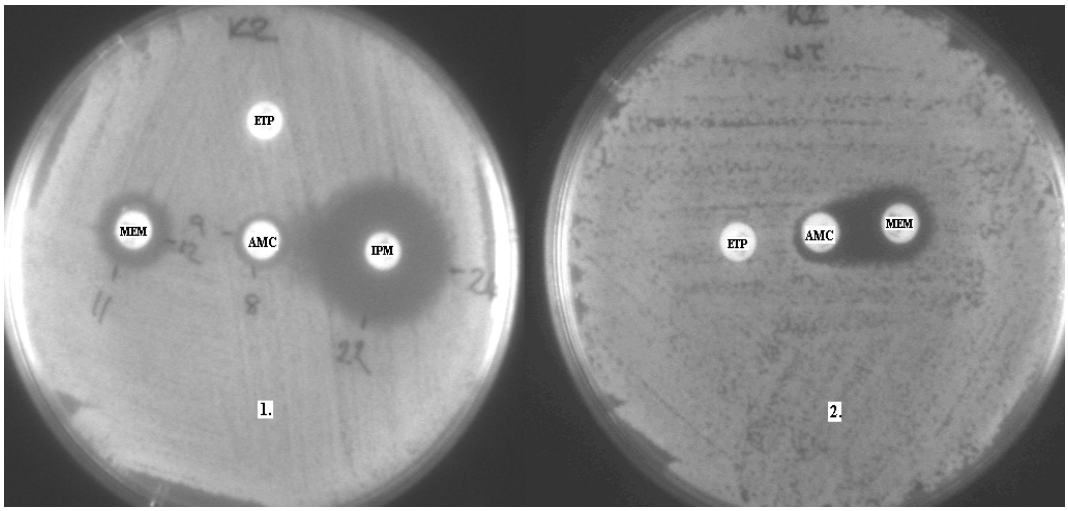


Figure 6.5. **Synergy disc testing of strain K2.** Synergy disc testing with ertapenem (ETP), imipenem (IMI), meropenem (MEM) and amoxicillin/clavulanic acid (AMC).

6.2.8 Modified Hodge test

A modified Hodge test was performed for both K1 and K2. *E. coli* ATCC 25922 was used as the indicator strain. This was performed using imipenem and meropenem antibiotic discs and a KPC-2 containing *K. pneumoniae* strain as a positive control. Neither strain exhibited the classical cloverleaf effect and so there were no phenotypic signs of carbapenemase production.

6.2.9 Phenotypic AmpC detection tests

Two phenotypic tests were performed to determine the presence of any AmpC enzymes in both K1 and K2; a modified three dimension test and an AmpC disc test.

Both assays showed no distortion of the zones of inhibition and so no AmpC enzyme was detected.

6.2.10 PCR screening of β-lactamases

Both K1 and K2 were screened by PCR for the presence of the following β-lactamase genes as listed in Table 6.4.

Class A	Found?	Variant	K1/K2
CTX-M	+	CTX-M-15	Both
GES	-		
IMI/SME	-		
KPC	-		
NMC	-		
PER	-		
SHV	+	SHV-1	Both
TEM	+	TEM-1	Both
VEB	-		
Class B			
GIM	-		
IMP	-		
NDM	-		
SIM	-		
SPM	-		
Class C			
AmpC	-		
VIM	-		
Class D			
OXA	+	OXA-1	Both

Table 6.4. **B-lactamase genes screened for in K1 and K2, those detected and the variants identified by sequencing.** + = found; - = not found.

All PCR products were sequenced and both strains were found to contain the following; CTX-M-15, OXA-1, SHV-1 and TEM-1 (Note: The PCRs of CTX-M,

OXA, SHV, TEM and AmpC, and subsequent sequencing was performed by A. Hamouda). Of note, no carbapenemases were detected in either strain and the absence of any *ampC* genes, as indicated by the phenotypic tests, was confirmed.

6.2.11 Iso-electric focussing (IEF)

IEF was attempted in order to identify any additional β-lactamases. The resulting IEF gels failed to show clearly focussed bands and so no conclusions could be drawn.

6.2.12 OMP extraction of K1 and K2

OMP extractions were performed from cultures grown in both LB broth (high osmolarity) and nutrient (low osmolarity) in order to distinguish between OmpK35 and OmpK36 as the former is inhibited in a high osmolarity medium. OMP extractions from K1 and K2 revealed that both strains were lacking a major band of ~40 kDa and additionally K2 lacked a major band of ~36kDa (Figure 6.6). These bands are presumed to be OmpK35 and OmpK36 respectively. OMP profiles were compared to those extracted from *K. pneumoniae* ATCC 13883, which has previously been reported to exhibit the full OMP complement and so was used as a control strain (Wu *et al*, 2001).

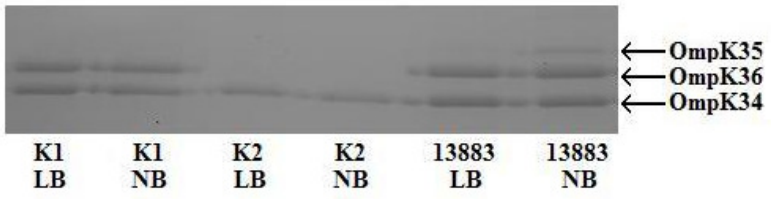


Figure 6.6. **SDS PAGE gel showing the major OMPs in strains K1, K2 and ATCC 13883.** The section of an SDS PAGE gel shows the expression of the three major OMP proteins in strain K1, K2 and ATCC 13883 when grown in both LB broth (LB) and nutrient broth (NB).

6.2.13 Sequencing of *ompK35* and *ompK36*

The entire ORFs of *ompK35* and *ompK36* from K1, K2 and *K. pneumoniae* ATCC 13883 were amplified and sequenced. Although the K1 and K2 OmpK35 and OmpK36 nucleotide and protein sequences were identical to one another, a number of differences were observed in the protein sequences between K1/K2 and ATCC 13883 (Figures 6.7 and 6.8, and Table 6.5). The differences observed in OmpK35 could be responsible for its absence on the SDS PAGE gels for both strains; however the absence of OmpK36 in K2 could not be attributed to the mutations found as both K1 and K2 have identical OmpK36 sequences.

OmpK35		OmpK36	
AA Position	**Change**	**AA Position**	**Change**
292	F/S	183	T → A
		184	L insertion
		192	G → T
		221	H → N
		225	N → L
		230	S insertion
		231	R → K
		233-234	DK → AL
		269-271	FSG → AGS
		272-278	7 AA deletion
		279	S → L
		314	I → L
		322	L → I
		353	D → S
		356-357	RR → HN

Table 6.5. **The changes in the OmpK35 and OmpK36 protein sequences from K1 and K2 relative to that observed in *K. pneumoniae* ATCC 13883.**

AA – amino acid; F/S – frame-shift.

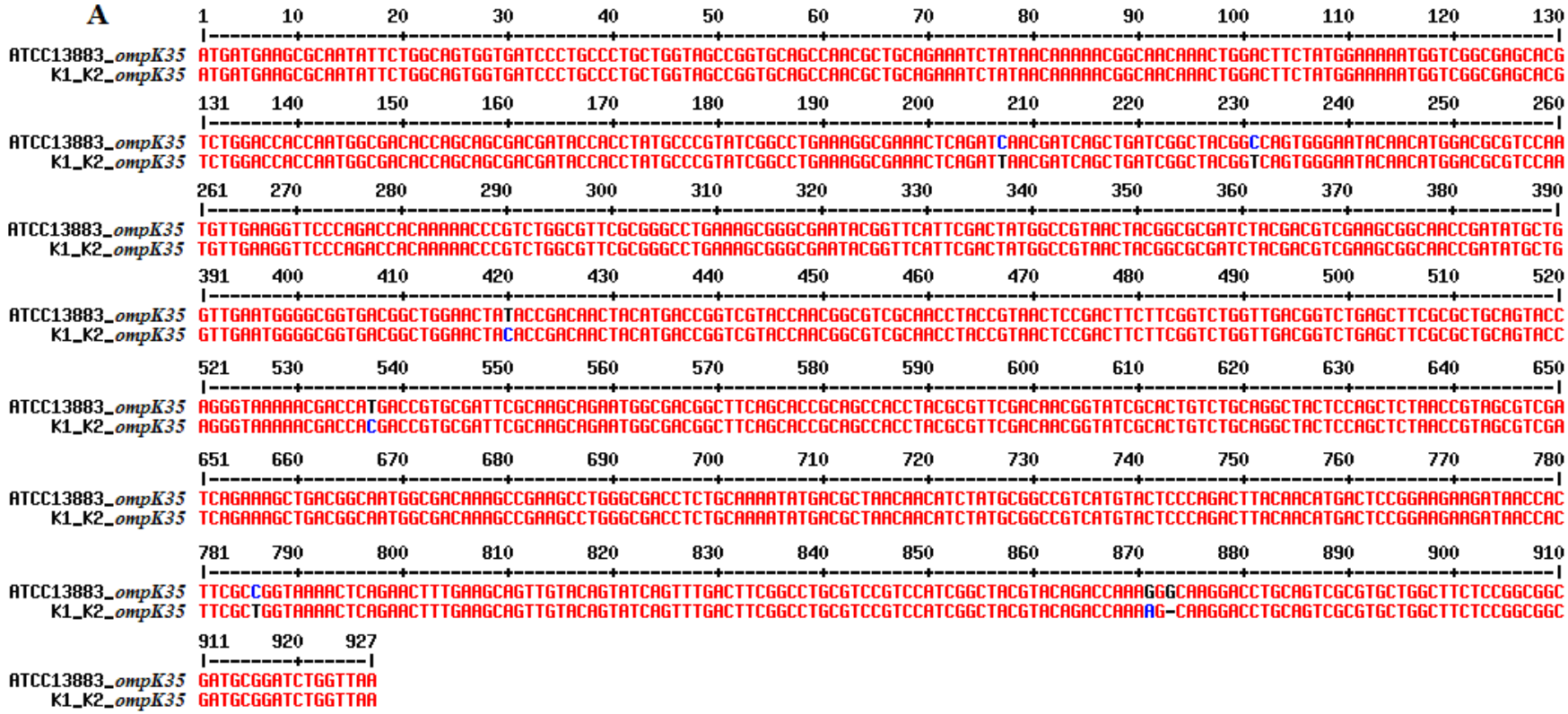


Figure 6.7A. **The nucleotide sequence of *ompK35* from K1 and K2 aligned with *ompK35* from ATCC 13883.** Note the single nucleotide deletion at position 873 in K1 and K2, resulting in the frame shift as seen in Figure 3B.

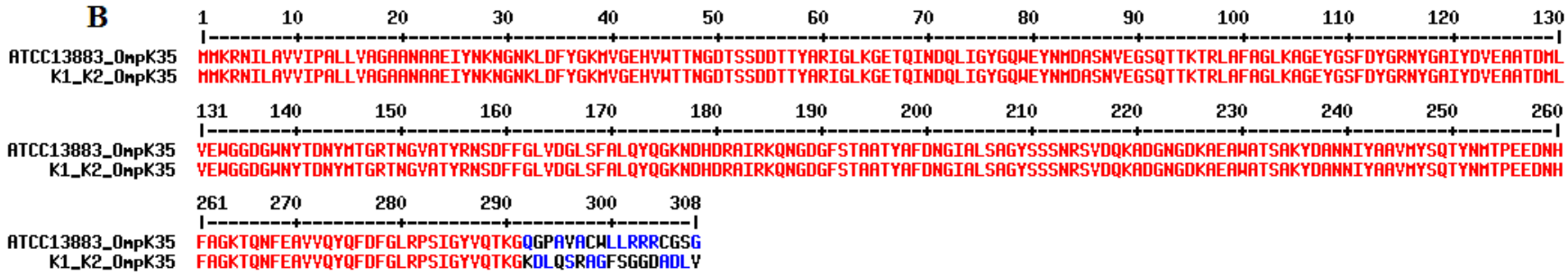


Figure 6.7B. **The protein sequences of OmpK35 from K1 and K2 aligned with OmpK35 from ATCC 13883.** Note the frame shift in K1 and K2 from position 292 onwards.

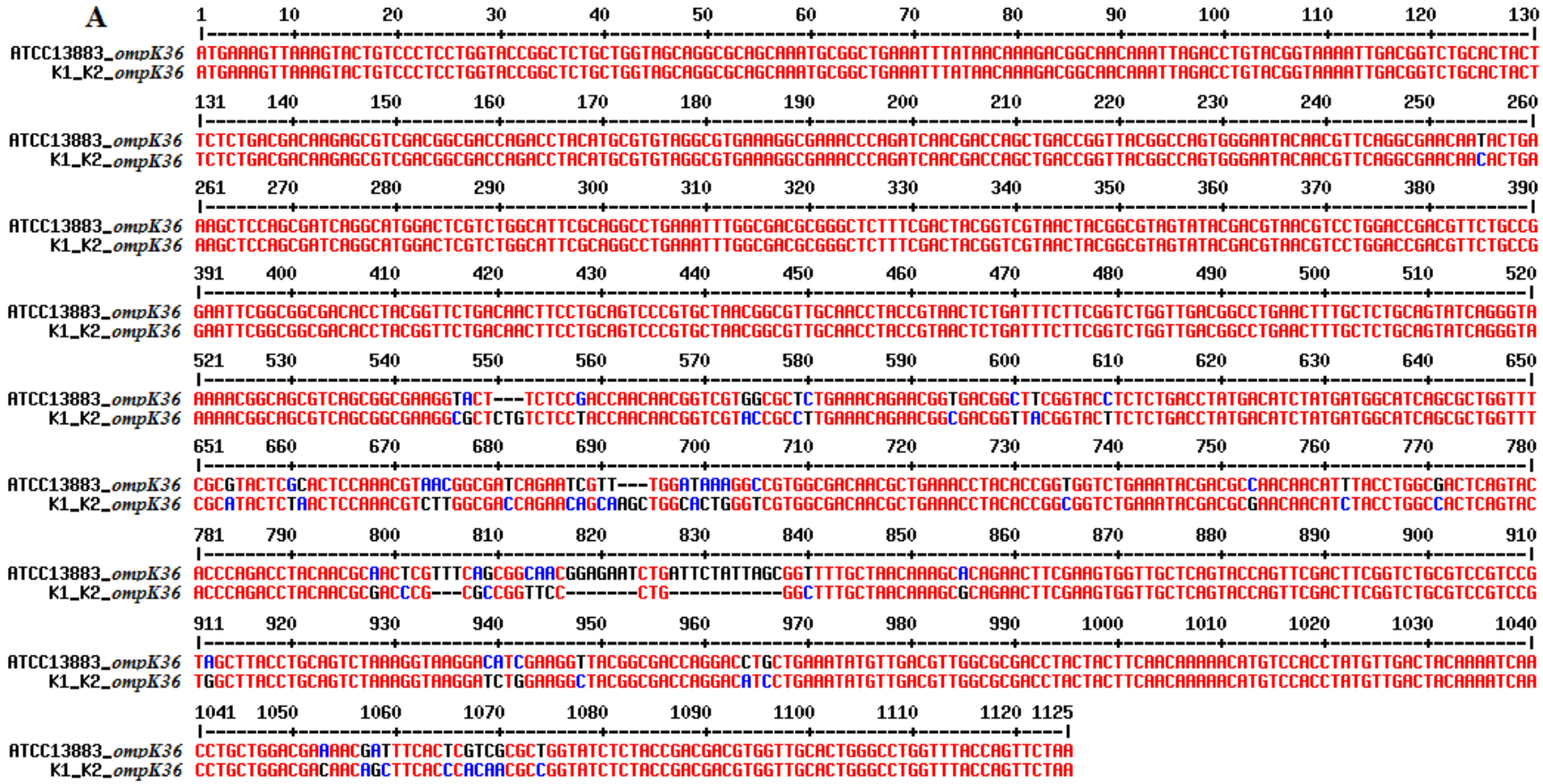


Figure 6.8A. **The nucleotide sequence of *ompK36* from K1 and K2 aligned with *ompK36* from ATCC 13883.**

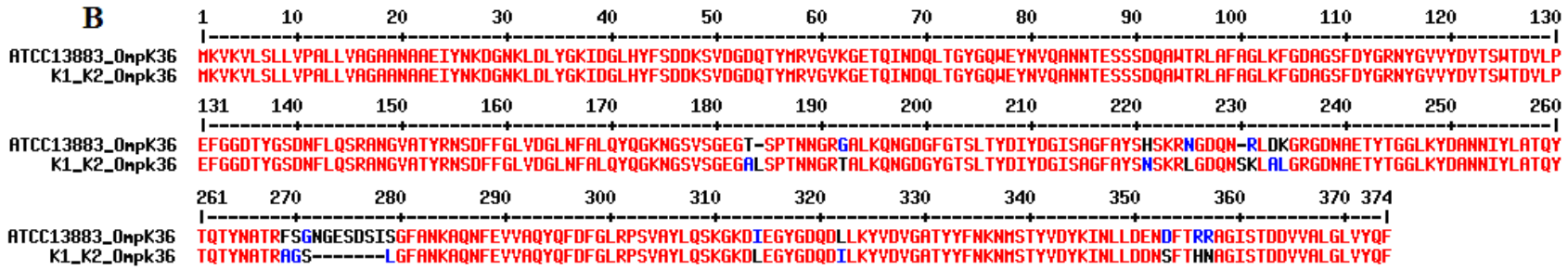


Figure 6.8B. **The protein sequences of OmpK35 from K1 and K2 aligned with OmpK35 from ATCC 13883.**

6.2.14 Carbapenem mutant studies

Carbapenem mutants of K1 were selected for using ertapenem, imipenem and meropenem. Mutant colonies were selected from plates containing the relevant antibiotic at 4 - 8 times their respective MICs. Concentrations of the antibiotics used and their mutation frequencies are shown in Table 6.6. Similar mutation studies were also attempted in K2 but failed to produce any mutants.

Antibiotic	MIC (µg/ml)	Antibiotic Conc.	Mutation Frequency
Ertapenem	1	4	7.74×10^{-8}
Imipenem	0.125	1	8.7×10^{-8}
Meropenem	0.5	4	1.74×10^{-8}

Table 6.6. **The mutation frequencies of K1 to ertapenem, imipenem and meropenem at the antibiotic concentrations used.**

The mutation frequencies were similar for each antibiotic with ertapenem and imipenem showing a slightly higher mutation frequency than meropenem but no increase that could be considered significant. Of the mutants obtained, four were chosen for each antibiotic for subsequent susceptibility testing, the results of which are shown in Table 6.7. The resistance profiles of the carbapenem mutants were highly similar to those observed in K2 for the carbapenem antibiotics. Additionally, the higher MICs of aztreonam and ciprofloxacin observed in K1 relative to K2, were maintained in the K1 mutants. Cefoxitin MICs in K1 mutants increased to that of K2. The only exception to this pattern was in the Imi_1-2 mutant which maintained carbapenem MICs more comparable to its K1 parent.

OMP extractions of the carbapenem mutants showed that the 36kDa porin loss, as observed in K2, was evident in 100% (4/4) of the picked ertapenem and meropenem mutants and in 75% (3/4) of the imipenem mutants. K1 Imi_1-2 was the only mutant that did not display the 36kDa porin loss, which was reflected in its considerably lower carbapenem MICs with respect to the other mutants. This suggests that the most significant, and perhaps even sole difference between K1 and K2 with regard to the carbapenem resistance phenotype, is the absence of the 36kDa porin. Subsequent attempts to select for second generation mutations were unsuccessful, suggesting that the levels of carbapenem resistance reached in the first generation mutants were at their optimum and therefore could not be increased.

	MIC (µg/ml)								
Strain	**Amikacin**	**Ampicillin**	**Aztreonam**	**Cefoxitin**	**Ceftazidime**	**Chloramphenicol**	**Ciprofloxacin**	**Colistin**	**Ertapenem**
K1	16	>512	>256	64	>128	64	>256	1	1
K2	16	>512	64	>128	>128	64	64	1	64
Imi_1-1	32	>512	>256	>128	256	64	>256	1	>16
Imi_1-2	32	>512	>256	32	128	64	>256	1	1
Imi_1-3	32	>512	>256	>128	256	64	>256	1	>16
Imi_1-4	32	>512	>256	>128	256	64	>256	1	>16
$Mero_4$-1	32	>512	>256	>128	256	64	>256	1	>16
$Mero_4$-2	16	>512	>256	>128	128	64	>256	1	>16
$Mero_4$-3	16	>512	>256	128	256	64	>256	2	>16
$Mero_4$-4	32	>512	>256	>128	256	64	>256	2	>16
Ert_4-1	32	>512	>256	>128	128	64	>256	1	>16
Ert_4-2	32	>512	>256	>128	256	64	>256	2	>16
Ert_4-3	32	>512	>256	>128	256	64	>256	2	>16
Ert_4-4	32	>512	>256	>128	256	64	>256	2	>16

Table 6.7A. **Susceptibility testing results of K1 carbapenem mutants compared to both K1 and K2.**

	MIC (µg/ml)							
Strain	**Gentamicin**	**Imipenem**	**Imipenem/EDTA**	**Meropenem**	**Piperacillin**	**Sulbactam**	**Tetracycline**	**Tobramycin**
K1	128	0.125	0.125	0.5	>512	>32	16	64
K2	128	2	2	8	>512	>32	8	64
Imi_1-1	128	2	1	8	>512	>32	8	128
Imi_1-2	64	1	1	0.125	>512	>32	8	128
Imi_1-3	128	2	1	8	>512	>32	8	128
Imi_1-4	128	2	1	8	>512	>32	8	128
$Mero_4$-1	128	2	1	8	>512	>32	16	256
$Mero_4$-2	128	2	1	8	>512	>32	16	256
$Mero_4$-3	128	2	1	8	>512	>32	16	64
$Mero_4$-4	128	2	1	8	>512	>32	16	128
Ert_4-1	128	2	1	8	>512	>32	16	128
Ert_4-2	128	2	1	8	>512	>32	16	128
Ert_4-3	128	2	1	8	>512	>32	16	128
Ert_4-4	128	2	1	8	>512	>32	16	128

Table 6.7B. **Susceptibility testing results of K1 carbapenem mutants compared to both K1 and K2.**

Ertapenem and meropenem mutant studies performed in strain MGH 78578 showed that, whilst it was possible to deplete the 36kDa OMP to give the same OMP profile as observed in K2, this did not result in significant carbapenem resistance comparable to the levels achieved in the K1 ertapenem mutants (Table 6.8). The MGH 78578 Ert mutant exhibited resistance to ertapenem, although at a much lower level than in the K1 Ert mutant, and just a modest reduction in susceptibility to meropenem. The MGH 78578 Mero mutant exhibited modest increases in susceptibility to all three antibiotics but again at a far lower level than the K1 mutant equivalent. This shows that the levels of resistance achieved to the carbapenems in K1 Ert and K1 Mero cannot be achieved in their MGH 78578 counterparts. These results suggest that the 36kDa OMP loss, only, cannot result in carbapenem resistance observed in K2 and that MGH 78578 must lack another resistance mechanism necessary for the phenotype observed in both K2 and the K1 mutants.

	MIC (µg/ml)					
	Imipenem	**S/I/R**	**Ertapenem**	**S/I/R**	**Meropenem**	**S/I/R**
MGH 78578	0.25	S	0.25	S	0.125	S
MGH 78578 Ert	0.25	S	4	R	0.5	S
MGH 78578 Mero	0.5	S	1	S	0.5	S
K1	0.125	S	4	R	0.125	S
K1 Ert	4	I	128	R	8	R
K1 Mero	4	I	128	R	8	R

Table 6.8. **A comparison of the carbapenem MICs of K1 ertapenem and meropenem mutants against MGH 78578 ertapenem and meropenem mutants.**

6.2.15 Stability of resistance phenotype and OMP loss in K1 carbapenem mutants

To determine whether the resistant phenotype to ertapenem, imipenem and meropenem in the carbapenem mutant experiments was stable, the strains were subcultured on IST agar for 10 days. The strains were then subject to susceptibility testing and their outer membrane proteins were extracted for analysis. Tested strains contained representatives of each of the generated carbapenem mutants (ertapenem, imipenem and meropenem). No differences were observed in the susceptibility or the OMP profiles of the subcultured strains and so the phenotypes were concluded to be stable.

6.2.16 Analysis of gene expression

RT-PCR was performed on strains K1, K2 and ATCC 13883 to determine the relative expression levels of the following: genes encoding the outer membrane proteins; *ompA, ompC, ompF,* and genes that have been associated with an MDR phenotype via the over-expression of the efflux pump AcrAB*; ramA, romA*, *acrA* and *16S,* the latter gene was used as an internal control. The strains were grown to exponential growth phase (OD_{600}~0.5) in both LB broth and nutrient broth respectively.

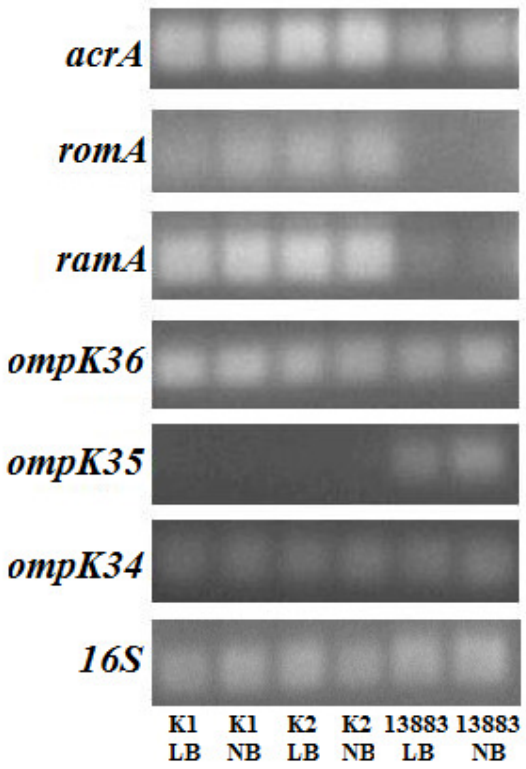


Figure 6.9. **Gel showing the PCR products as amplified from K1, K2 and ATCC 13883 cDNA for the genes of interest.**

As shown in Figure 6.9, it appears as though *ompK35* was not expressed in either K1 or K2 which correlates with its absence in their OMP profiles. *ompK36* however appeared to have been expressed in both strains despite the absence of OmpK36 in the OMP profile of K2. It is also evident that both genes *ramA* and *romA* are over-expressed in K1 and K2 relative to the levels observed in ATCC 13883, and *acrA* expression also appeared to be more prominent in K1 and K2, correlating with the results of the earlier study on the *ramA* regulon. *ompK34* has not been associated with the conferring of antibiotic resistance and *16S* is a constitutively expressed gene, and so are shown as internal controls.

6.2.17 Sequencing of *ramR*

Upon the recognition that *ramA* was over-expressed in both K1 and K2, its local regulator gene, *ramR*, was amplified and sequenced. The sequence was compared to that of *K. pneumoniae* strain MGH 78578, which has previously been shown to be a *ramA* non-expresser and whose *ramR* can be considered to be of the wild-type sequence.

Sequence analysis revealed that K1 and K2 had identical *ramR* nucleotide sequences and contained three changes relative to the MGH 78578 sequence (Figure 6.10A). The protein sequence alignments (Figure 6.10B) show that the nucleotide change at position 579 is silent, but the changes at positions 57 and 247 result in the following; A19V and E83stop. The presence of the stop codon in the *ramR* of both strains can be presumed to result in a non-functional RamR protein resulting in the subsequent over-expression of *ramA*.

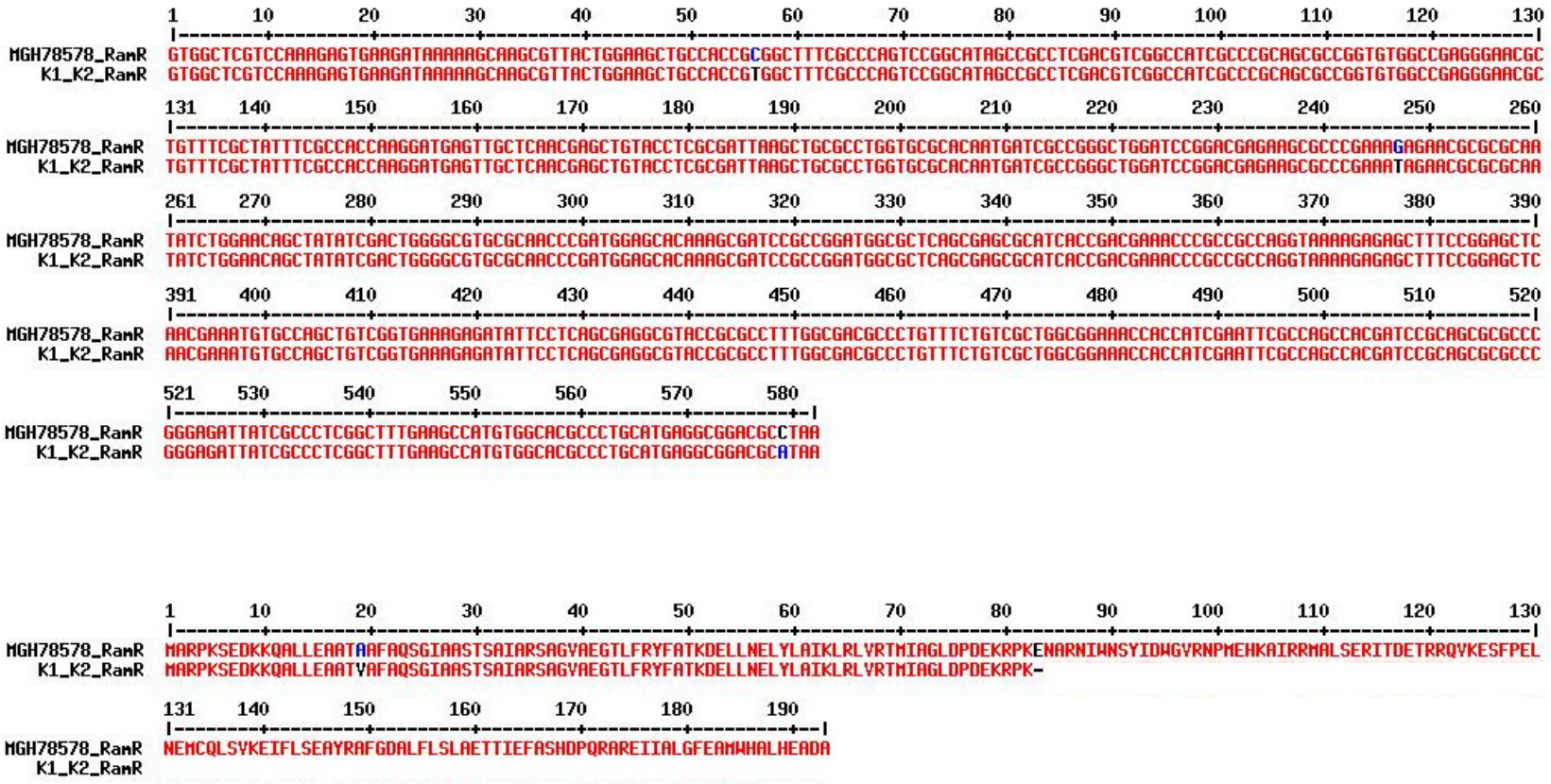


Figure 6.10. **The nucleotide (A) and protein sequences (B) of *ramR*/RamR from K1, K2 against the wild-type sequences from MGH 78578.**

6.2.18 Plasmid profiling of K1 and K2

Plasmids were extracted from K1, K2 and also *K. pneumoniae* MGH 78578 (which contains plasmids of known sizes) and run on an agarose gel overnight (Figure 6.11).

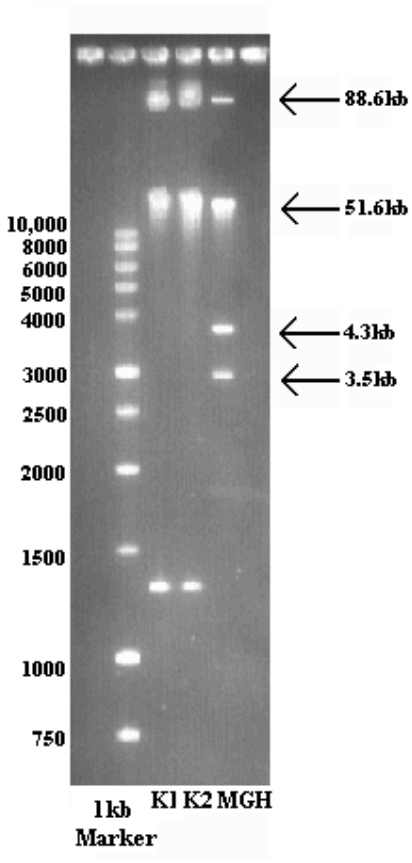


Figure 6.11. **An agarose gel showing the plasmid profiles of K1, K2 and MGH 78578.** A 1kb DNA ladder with the DNA band sizes (in bp) are noted on the left and the sizes of known plasmids in MGH 78578 are noted on the right.

In the agarose gel both K1 and K2 appear to contain three plasmids of similar sizes, although true sizes cannot be accurately determined whilst the plasmids remain in

their native, circular forms. No obvious differences between the two strains can be ascertained from these results. Plasmids were further profiled by S1 PFGE, which should allow the determination of true plasmid sizes. K1, K1S, K2 and K2S were analysed by this technique (Figure 6.12).

K1 and K1S plasmid profiles appear to be identical, both of which showing four plasmids. K2 and K2S however showed differences in their plasmid profiles: K2S appears to lack the plasmid of around 70kb, and instead has gained another plasmid of <48kb. Additionally K1 appears to contain an extra plasmid of <48kb with regard to K2 (Figure 6.12 and Table 6.9) As the only difference between K2 and K2S was that K2S was subject to subculturing for ten days, it is highly unlikely that this is truly an additional plasmid. It is possible that the plasmid of around 70kb has instead lost a large section of its DNA as a result of plasmid instability and now appears smaller in size. It could be hypothesised that the missing section of plasmid may contain an aminoglycoside resistance determinant, as resistance profiles to the aminoglycoside antibiotics were the only differences observed between K2 and K2S.

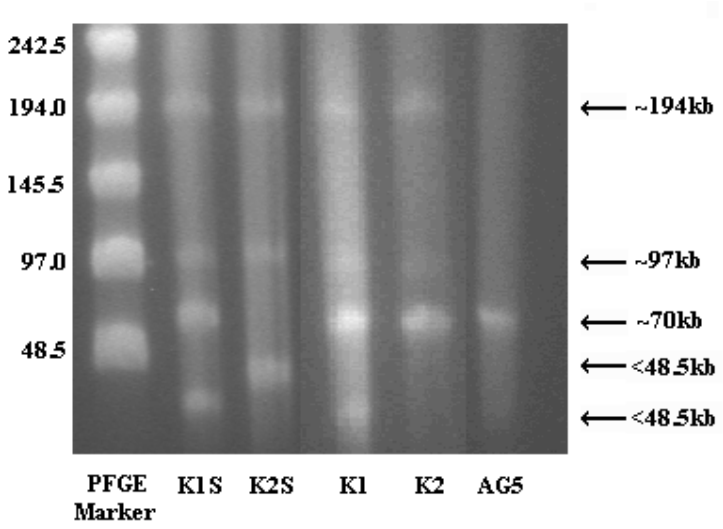


Figure 6.12. **An agarose gel showing the plasmid profiles of K1, K1S, K2, K2S and AG5 (AG5 - section 6.2.19).**

	No of Plasmids	Sizes (kb)
K1	4	194, 97, 70, <48
K2	3	194, 97, 70
K1S	4	194, 97, 70, <48
K2S	3	194, 97, <48
AG5	1	70

Table 6.9. **The number of plasmids and approximate sizes present in the parent, subcultured and transconjugant strains.**

6.2.19 Plasmid curing

Plasmid curing was attempted in both K1 and K2 using the curing agents; ethidium bromide, SDS and acriflavine. Curing success was assessed by susceptibility testing of 20 'cured' colonies for each agent. The colonies were tested against imipenem,

gentamicin and ceftazidime. No changes in MICs were observed for any of the 'cured' colonies and so it was concluded that no plasmid containing genes that affect the MICs of the tested antibiotics were lost from the strains.

6.2.20 Transconjugation studies

Transconjugation experiments were set up with K1 and K2 as donors and rifampicin resistant *E. coli* J62-2 as the recipient strain. Susceptibility testing was performed on suspected transconjugants as shown in Table 6.10. Carbapenem resistance was non transferable to J62-2. Gentamicin, tobramycin and ceftazidime resistance were all successfully transferred from K1 into J62-2. No transconjugants were obtained from K2.

	MIC (µg/ml)				
	Ceftazidime	**Cefoxitin**	**Gentamicin**	**Imipenem**	**Tobramycin**
Range Tested	0.0625-128	0.0625-512	0.0625-512	0.0625-32	0.0625-512
K1	128	64	128	0.125	64
K2	128	>128	128	2	64
E. coli J62-2	<8	<4	0.25	0.125	<2
AG1	64	<4	64	0.25	32
AG2	64	<4	64	0.25	32
AG3	64	<4	64	0.25	64
AG4	64	<4	64	0.25	128
AG5	64	<4	64	0.25	128
AG6	64	<4	64	0.25	64
AG7	64	<4	64	0.25	64
AG8	64	<4	64	0.25	64
AG9	64	<4	64	0.25	64
AG10	64	<4	64	0.25	64

Table 6.10. **Susceptibilities of *E. coli* J62-2 transconjugants (AG1-10) compared to those of K1, K2 and *E. coli* J62-2.**

6.2.21 Transconjugant J62-2 AG5

The transconjugant strain appears to have acquired just one plasmid from K1 of around 70 kb in size as shown by S1 PFGE (Figure 6.12). This same plasmid appears to be absent from the K2S strain. The gain of this plasmid in J62-2 AG5 and loss in K2S correlates with the MIC data in Table 6.9 and the assumption that the plasmid contains a gene capable of conferring aminoglycoside resistance.

- 70kb Plasmid

PCRs were performed on strains K1, K2, K1S, K2S, J62-2 and J62-2 AG5 for the following genes: CTX-M, OXA, SHV and TEM.

	Genes			
Strain	**CTX-M**	**OXA**	**SHV**	**TEM**
K1	+	+	+	+
K2	+	+	+	+
K1S	+	+	+	+
K2S	+	-	+	+
J62-2	-	-	-	-
J62-2 AG5	+	+	-	+

Table 6.11. **β-lactamase genes detected by PCR.** + = found; - = not found.

The data in Table 6.11 suggests that the ~70 kb plasmid contains CTXM-15, OXA-1 and TEM-1 in addition to the gene conferring aminoglycoside resistance. The lack of OXA-1 in K2S suggests that this gene is located in the same region of DNA that is presumed to contain the aminoglycoside resistance gene, which has subsequently

been lost. SHV-1 is likely to be chromosomally encoded in K1 and K2 as is found in many *K. pneumoniae* isolates, and therefore it has not been transferred to the transconjugant strains.

6.3 Discussion

6.3.1 Aim of this work

The work in this chapter was performed with the purpose of characterising the strains K1 and K2, establishing their relationship to one another, and ascertaining the mechanism(s) by which they exhibit an MDR phenotype, particularly to the carbapenem antibiotics.

6.3.2 β-lactam and carbapenem resistance mechanisms

Mechanisms of carbapenem resistance have become an important area of study in members of the Enterobacteriaceae as well as for other species of bacteria including *P. aeruginosa* and *A. baumannii*. Of particular importance is the prevalence and spread of carbapenem resistant strains within the hospital environment and patients. This causes significant problems for the success of antibiotic therapy. Carbapenem resistance in *K. pneumoniae* is most commonly mediated by the production of carbapenemases, which act to facilitate the hydrolysis of the β-lactam ring and therefore disable the carbapenem antibiotics. An increasing number of carbapenmases have been identified in *K. pneumoniae*, most recently the KPC and NDM enzymes in 2001 and 2009 respectively (Yigit *et al*, 2001; Yong *et al*, 2009). Since their initial discoveries in the USA and India, incidences of both enzymes have been reported worldwide (Struelens *et al*, 2010; Babouee *et al*, 2011; Mulvey *et al*, 2011; Poirel *et al*, 2011; Zhang *et al*, 2011). Additionally, the production of non-

carbapenemase β-lactamases, particularly the *ampC* genes, in conjunction with major OMP loss and active efflux mechanisms have been associated with a carbapenem resistant phenotype (Kohler *et al*, 1999, Quale *et al*, 2006, Yang *et al*, 2010). The conferring of carbapenem resistance is a complex area of research often incorporating a multitude of mechanisms rather than being attributable to just one.

A number of factors, including the identical PFGE profiles and their isolation in close proximity from a single patient, make it highly likely that strains K1 and K2 are isogenic. The differences observed in the levels of biofilm produced and the growth curves may be indicative of a 'fitness cost' as a consequence of the levels of carbapenem resistance conferred by K2. Both of these show a marked difference between the two strains.

The levels of MDR exhibited by both strains indicate the presence of a number of resistance mechanisms. The levels of resistance to the cephalosporin antibiotics could be attributed to the presence of the β-lactamases CTX-M-15, SHV-1 and TEM-1, which are capable of conferring the levels of cephalosporin resistance observed. The over-expression of the transcriptional activator *ramA*, as a result of a stop codon within the *ramR* regulator gene, indicates that it is likely that the elevated expression of efflux pumps are, in part, responsible for the MDR phenotype. It is plausible that this mutation was responsible for the resistance exhibited to the fluoroquinolones, tetracycline and tigecycline, as earlier studies suggest that these antibiotics are substrates of the RND family efflux pumps commonly associated with regulation by RamA (Elkins and Nikaido, 2002; Schneiders *et al*, 2003). The lack of

carbapenemase gene detection, despite extensive screening by PCR and a modified Hodge test, denotes that such a gene is unlikely to play any role in the carbapenem resistance phenotype. Additionally the lack of *ampC* detection by both PCR and phenotypic assays indicate that an AmpC enzyme does not contribute to the carbapenem resistant phenotype.

The expression of the major OMPs has been associated with resistance to the β-lactam antibiotics and indicated as a contributory factor in carbapenem resistance (Yang *et al*, 2009; Yang *et al*, 2010). The OMP profiles of K1 and K2 revealed a key difference between the strains; K2 did not express OmpK36. Expression of the major OMPs are certainly important factors for the permeation of various classes of antibiotics across the bacterial cell membrane and it appears that in *K. pneumoniae*, OmpK36 is of particular importance for permeability of the carbapenems. The results of the K1 carbapenem mutant studies suggest that K1 is indeed the progenitor of K2, and that meropenem therapy is likely to have selected for the carbapenem resistant phenotype *in vivo*. The susceptibility testing and OMP analysis of the carbapenem mutants show that the levels of resistance and OMP profile exhibited by K2 could be achieved in K1 by using any of the three carbapenems tested as a selective agent. There are however some aspects of the K2 susceptibility profile that could not be explained by selection with the carbapenem antibiotics. Both aztreonam and ciprofloxacin MICs were >256μg/ml in K1 but 64μg/ml in K2, and the K1 carbapenem mutants maintained the higher MICs of the K1 parent strain. Although the MICs displayed by both K1 and K2 render the strains resistant to aztreonam and

ciprofloxacin, it remains unknown what causes the reductions in MICs to these antibiotics that were achieved *in vivo* but not *in vitro*.

The results of the carbapenem mutant studies in strain MGH 78578 suggest that the loss of OmpK36 affects the MICs as follows: Ertapenem > Meropenem > Imipenem. The greatest reductions in susceptibility following OmpK36 loss are observed for ertapenem (4-16-fold), then meropenem (4-fold) and modest changes for imipenem (2-fold). It has been shown that imipenem is capable of penetrating the bacterial cell wall faster than meropenem, likely due to a combination of its zwitterionic charge and smaller size, and these differences may allow imipenem to penetrate other porins that ertapenem and meropenem cannot (Yang *et al*, 1995). The results of the K1 carbapenem mutant studies on the other hand show markedly different results. All three types of mutants (ertapenem, imipenem and meropenem) exhibited the same reductions in susceptibility with 64-fold changes for both ertapenem and meropenem, and a 32-fold change for imipenem, suggesting that the porin loss has the following effect on MICs; ertapenem = meropenem > imipenem. The vast difference in susceptibility changes between MGH 78578 and the K1 carbapenem mutants suggest that although OmpK36 loss is an important factor in conferring resistance, additional mechanisms present in K1 but absent in MGH 78578 are also necessary to confer the phenotype observed. In order to decipher the causes of the carbapenem resistant phenotype of K2, it is essential that these additional mechanisms are identified.

It has been suggested that non-carbapenemase ESBLs may play a role in conferring resistance to the carbapenems, particularly ertapenem. Although non-carbapenemase

ESBLs are noted for their ability to hydrolyse cephalosporins, none have been definitively shown to have the ability to confer resistance to the carbapenems. It has previously been suggested that CTX-M-15 may be capable of contributing towards carbapenem resistance through the hydrolysis of ertapenem (Girlich *et al*, 2008). Girlich *et al* (2008) showed that CTX-M-15 exhibited higher β-lactamase activity against ertapenem than other CTX-M enzymes and TEM-3, although its activity was still substantially lower than that of the carbapenemase KPC-2 (1.6mU/mg vs. 98mU/mg). Compared to the wild-type *K. pneumoniae* ATCC 13883, K1 exhibits reduced susceptibility to ertapenem (0.125µg/ml vs. 1µg/ml) indicating that K1 may already be predisposed to resistance even before exposure to any carbapenem antibiotics. The fact that both strains K1 and K2 contain CTX-M-15, suggest that this enzyme may be involved in conferring ertapenem resistance, in line with the findings of another study (Girlich *et al*, 2009). The other β-lactamases identified in strains K1 and K2 (OXA-1, SHV-1, TEM-1) are highly unlikely to play any role in conferring any ertapenem resistance as none of these have ever been reported to possess any carbapenemase activity and additionally strain MGH 78578, which contains TEM-1, and *E. coli* control strains containing TEM-1 and SHV-1, were completely sensitive to ertapenem. Similarly, the carbapenem mutants of both MGH 78578 and K1 exhibited completely different levels of resistance to ertapenem as well as meropenem and imipenem, despite the same resulting OMP profiles. It must also be considered that despite the detection of just one ESBL in these strains (CTX-M-15) it is possible that they additionally contain more than one SHV or TEM-type β-lactamase, and that only the predominant narrow-spectrum β-lactamases (SHV-1 and TEM-1) were shown by sequencing.

The production and dissemination of carbapenemases is often seen as the primary cause of carbapenem resistance and subsequently the incidences of carbapenemases is closely monitored in the UK by the HPA. However, this study shows that levels of carbapenem resistance, sufficient to result in therapy failure, are possible in the absence of such enzymes. A combination of resistance mechanisms, that are seemingly insignificant in regard to the conferring of carbapenem resistance singly, can result in resistance together. This complex interplay between different resistance mechanisms is a particularly worrying phenomenon as they are a great deal more difficult and time consuming to identify compared to the presence of single resistance genes such as those encoding carbapenemases. Additionally, as was observed with these strains, the elevated levels of resistance observed in strain K1 was only subtly more than that of wild-type *K. pneumoniae* strain, MGH 78578, and so the predisposition to becoming carbapenem resistant upon exposure to the antibiotics may not be easily phenotypically detectable.

The contribution of efflux mechanisms to carbapenem resistance is currently a disputed topic with some studies reporting a reduction in susceptibility to the carbapenem antibiotics upon the activation of efflux (Kohler *et al*, 1999; Szabo *et al*, 2006), whilst other studies report little or no effect (El Amin *et al*, 2005; Kaczmarek *et al,* 2006). Despite any foregone conclusions regarding the ability of efflux to contribute to carbapenem resistance, it remains clear that the presence of active efflux diminishes the viability of other antibiotic treatment options and so the capacity of efflux mechanisms should not be discounted. Whilst in this study, only the expression of the efflux pump AcrAB was investigated, it would be imprudent to

disregard the possibility of AcrAB contributing to carbapenem resistance or the presence of other efflux systems, that may be involved in conferring the phenotype.

6.3.3 Plasmid analysis

Plasmid-mediated transfer of resistance genes can be considered to be one of the most important mechanisms for the spread of antibiotic resistance. In *K. pneumoniae,* plasmid-mediated resistance has been associated with the spread of genes conferring resistance to several classes of antibiotics including the fluoroquinolones and aminoglycosides, but particularly the β-lactams via the dissemination of genes encoding β-lactamases. The acquisition of resistance plasmids, plasmids that carry two or more resistance determinants, provide a direct mechanism by which a bacterium can become resistant to multiple classes of antibiotics.

The transconjugation studies and plasmid profiling showed that the β-lactamase genes CTX-M-15, OXA-1, and TEM-1 are present on the same plasmid whilst SHV-1 is likely to be chromosomally located, as is typical in *K. pneumoniae* (Leung *et al*, 1997). The transfer of aminoglycoside resistance indicated that this plasmid additionally carries an aminoglycoside resistance determinant. The loss of aminoglycoside resistance in strain K2S, suggests that this gene has been lost from the plasmid and the results of S1 PFGE show that the plasmid is significantly smaller in size. This is likely as a result of plasmid instability, where unnecessary regions of

DNA can be deleted and plasmids ends can recombine to create smaller, more compact plasmids.

The loss of plasmid-resistance markers has been documented in *S. enterica*, where it was shown that in the absence of antibiotic pressure, resistance is lost whilst the plasmid itself remains (Mendoza-Medellin *et al*, 2004). In the case of K2 and KS it appears that a large section of the plasmid DNA has been lost rather than only the gene encoding aminoglycoside resistance. It was also shown that OXA-1 was lost, indicating that it likely resided in the same region of the plasmid. Large plasmid DNA losses are not an undocumented phenomenon. Williams *et al* (1988) observed a 39kb deletion of DNA from a plasmid in *P. putida* that encoded genes required for the catabolism of toluene and xylene. It has been shown that the maintenance of large plasmids incurs a fitness cost on the bacterium and strains that lose plasmids as a result of segregational instability, can grow more rapidly than their parental counterparts, eventually taking over the population (Smith and Bidochka, 1998). It could be hypothesised that K2S lost a large region of its plasmid as a mechanism of reducing costs on fitness.

6.3.4 Conclusions

It could be hypothesised that reduced susceptibility to the carbapenem antibiotics can be achieved through a plethora of contributory mechanisms, which on their own have no significant effect but together can confer levels of resistance to the carbapenems sufficient to result in the failure of therapy. As shown in this work, exposure of the

bacterium to ertapenem, imipenem or meropenem results in a universal effect of OmpK36 porin loss, the result of which is reduced susceptibility across the carbapenem antibiotics. As ertapenem is typically used for the treatment of community infections, it is important to appreciate that ertapenem usage prior to usage of meropenem may inadvertently select for reduced susceptibility and cause therapy failure in strains that are predisposed to becoming carbapenem resistant. The results of this study compound the increasing threat of carbapenem resistance and show that it is not only carbapenemases that require close monitoring under resistance surveillance studies, but additionally other ESBLs, such as CTX-M-15 (which is the most commonly isolated of the CTX-M enzymes in the UK (Livermore *et al*, 2007)), must not be forgotten or overlooked as being less of a threat. This indicates that the practice of conserving the carbapenem antibiotics until absolutely necessary is more important than ever as the availability of other antibiotics is a dwindling option.

7. Discussion and conclusions

K. pneumoniae has recently been the focus of media attention regarding the spread of strains containing the carbapenemase NDM-1 and the problems associated with treating such infections (Struelens *et al*, 2010). Fortunately such strains are still relatively rare in the UK although their emergence does demonstrate that *K. pneumoniae* is a bacterium capable of constantly acquiring new antibiotic resistance mechanisms. Since the emergence and dissemination of β-lactamases in *K. pneumoniae*, few treatment options remain viable. Presently the carbapenem antibiotics are considered the 'antibiotics of last resort' for complicated *K. pneumoniae* infections and those that harbour ESBLs; however even these compounds are now in danger of becoming ineffective due to the rapid evolution and dissemination of carbapenemases, such as NDM-1.

Treatment options for infections caused by *K. pneumoniae* are dependent upon a number of factors including: the site of infection, any current medications, and previous antibiotic therapy. Typically the aminoglycosides, fluroquinolones and penicillins/β-lactamase inhibitor combinations are the first line antibiotics used in the treatment of infections followed by the cephalosporins, all of which can be utilised in either monotherapy or combination therapy. In the case of ESBL-producing strains the carbapenem antibiotics are the preferred choice, although these can be inadequate in the presence of strains harbouring carbapenemases. The glycylcycline antibiotic, tigecycline, can be utilised in the treatment of carbapenem-resistant infections, however its rapid diffusion into the tissues from serum and low concentration in the

urine mean it is limited in its ability to treat these infections and as such requires close monitoring (Peleg and Hooper, 2010). *K. pneumoniae*, however, is capable of conferring resistance to all of these compounds through a plethora of mechanisms and as such presents a dilemma.

The main focus of this PhD was to investigate the mechanisms of antibiotic resistance employed by clinical and environmental strains of *K. pneumoniae*.

This study has shown that the transcriptional activator RamA is an important factor in the conferring of resistance to antibiotics that are subject to efflux via the AcrAB efflux pump. RamA over-expressing strains were shown to exhibit elevated MICs to various classes of antibiotics including chloramphenicol, the fluroquinolones and the tetracyclines. It has previously been shown that the upregulation of *ramA* transcription is associated with the upregulation of *acrA*, a gene encoding a component of the RND family efflux pump, AcrAB (Schneiders *et al*, 2003). Two chemicals, chlorpromazine (CPZ) and tigecycline, were shown to upregulate genes deemed to be within the *ram* regulon; *ramA*, *romA* and *acrA*. These findings suggest that these chemicals are capable of selecting for the upregulation of *ramA*, which in turn upregulates *acrA,* resulting in increased efflux capabilities. This is an important finding as the 'switching on' of this single mechanism is sufficient to lead to an MDR phenotype that can result in the failure of therapy to many of the first line antibiotics used to treat *K. pneumoniae* infections. The ability of CPZ to exhibit synergistic effects with chloramphenicol, norfloxacin and tetracycline is contrasted by its ability to upregulate *ramA* and potentially select for efflux mutants. Although

the phenothiazines may have a potential for clinical use, it is apparent that this may be a double-edged sword, where they may also be detrimental to the viability of other antimicrobials. The upregulation of *ramA* over-expression by tigecycline is a particularly significant and worrying finding as tigecycline is an antibiotic reserved to treat complicated *K. pneumoniae* infections and its ability to select for an MDR phenotype brings into question the appropriateness of its use. The effects of both CPZ and tigecycline in the upregulation of *ramA* and *acrA* suggest that *acrA* upregulation by both compounds is mediated by RamA, suggesting that the compounds themselves directly act upon either *ramA* itself or indirectly act upon its regulator(s).

The endophytic *K. pneumoniae* strain, Kp342 was shown to exhibit a MDR phenotype and analysis of its *ramR* gene sequence revealed a 32 amino acid deletion. Complementation with a wild-type *ramR* resulted in increased susceptibility to several antibiotics that are normally associated with efflux through the AcrAB efflux pump. Additionally the transcription of *ramA*, *romA* and *acrA* was shown to be downregulated in the *ramR* complemented strain relative to its parental counterpart. This suggests that RamR acts as a negative regulator of RamA and the deletion observed in its gene sequence is responsible for the over-expression of *ramA* and subsequently *acrA*, resulting in reduced susceptibility to several classes of antibiotics that are substrates of the AcrAB efflux pump. The deletion suggests that efflux is an essential mechanism for survival in Kp342, possibly as a means to cope with toxic substrates encountered in its environmental niche. The MDR phenotype exhibited by Kp342 may signify its potential threat as a human pathogen, however animal

infection models have shown that Kp342 is of lower virulence than its clinical counterparts, suggesting it is adapted specifically as an endophyte (Fouts *et al*, 2008). It was also observed that in this particular strain the complementation of a wild-type *ramR* resulted in a significant increase in biofilm production, suggesting a role for RamA in virulence.

Further investigation into the *ramR* gene sequences in a panel of *K. pneumoniae* containing both expressers and non-expressers showed a correlation between mutations within *ramR* and the over-expression of *ramA*. This suggests that RamR is involved in the negative regulation of RamA, and mutations within the *ramR* gene sequence are adequate to result in *ramA* over-expression. It was shown that CPZ, ciprofloxacin and tigecycline mutants of sensitive *K. pneumoniae* strain Ecl8, contained mutations within their *ramR* gene sequences. These mutants exhibited elevated MICs across several antibiotic classes correlating with an efflux mutant phenotype. The apparent targeting of these mutations towards the *ramR* gene is significant as both ciprofloxacin and tigecycline are known substrates of the AcrAB efflux pump, and CPZ is also likely to be subject to efflux. Complementation with a wild-type *ramR* in the mutant strains resulted in a decrease in MICs to the tested antibiotics, however not to the parental phenotype in all strains. These results suggest that although RamR is an important factor in the regulation of RamA, there are also other mechanisms involved in its regulation that remain to be identified.

The final study in this PhD focussed on the mechanisms of carbapenem resistance in two clinical strains of *K. pneumoniae*. It was shown that two strains of *K.*

pneumoniae, obtained from the urine of a single hospitalised patient on separate occasions, were isogenic. The patient had been treated with meropenem between the isolation of each of the strains. The strains differed only by their susceptibilities to the carbapenem antibiotics. The first obtained strain, K1 was carbapenem sensitive, and the subsequently obtained strain, K2, exhibited reduced susceptibility to the carbapenems. Analysis of the OMP profiles of both strains revealed that K2 lacked a protein of ~38kDa. Carbapenem mutation studies in K1 showed that the susceptibility phenotype and OMP profile exhibited by K2 could be achieved in K1 via a single step mutation using either ertapenem, imipenem or meropenem. This suggest that K1 was the progenitor of K2 following the *in vivo* exposure to meropenem during therapy. PCR analyses revealed that both strains contained the β-lactamases OXA-1, SHV-1 and TEM-1 and the ESBL - CTX-M-15. Importantly neither strain contained any genes encoding carbapenemases or AmpC enzymes, both of which are commonly associated with reduced susceptibility to the carbapenem antibiotics. Further analysis of the OMPs showed the porin found lacking in K2 was OmpK36 and additionally both K1 and K2 lacked OmpK35. Susceptibility profiling identified that the loss of OmpK36 corresponded to reduced susceptibility to both ertapenem and meropenem, suggesting that OmpK36 acts as a point of entry for these antibiotics in to the cell. RT-PCR analysis showed that in both strains *ramA* and *acrA* was over-expressed suggesting the involvement of active efflux in the MDR phenotype. Comparative susceptibility testing in β-lactamase containing control strains in the presence of clavulanic acid showed that the ESBL CTX-M-15 may have a role in conferring resistance to the carbapenems. It was concluded that strain K2 had arisen from K1 as a direct result of meropenem therapy

and that the reduced susceptibility to carbapenem phenotype was conferred by a combination of mechanisms including porin loss, active efflux and the presence of CTX-M-15. This study indicated the plethora and complexity of mechanisms that can be employed by a single strain to confer an MDR phenotype.

The following conclusions were made with regard to the results of these studies.

- RamA over-expression correlates with the upregulation of the efflux pump AcrAB and subsequently a MDR phenotype in agreement with previous literature (Schneiders *et al*, 2003; Ruzin *et al*, 2005; Kallmann *et al*, 2008; Zheng *et al*, 2009).

- RamR acts as a negative regulator of RamA and subsequently plays a pivotal role in the regulation of AcrAB mediated efflux.

- Carbapenem resistance can be conferred in the absence of carbapenemase or AmpC enzymes.

- The ESBL CTX-M-15 plays a contributory role in conferring resistance to ertapenem when coupled with porin loss and active efflux.

The results of these studies show the multitude of mechanisms that can be employed by *K. pneumoniae* to become resistant to antibiotics. *K. pneumoniae*, similar to many other Gram-negative species, has the ability to rapidly acquire resistance

through a number of mechanisms when challenged. The acquisition of resistance genes such as β-lactamases, *qnr* genes, aminoglycoside resistance determinants, alteration of the permeability of the outer membrane through either porin loss or downregulation, and active efflux mechanisms are but a few mechanisms by which *K. pneumoniae* can confer resistance to most antibiotics. For example, National Nosocomial Infections Surveillance data reported that in 1986 that ~2% of *K. pneumoniae* strains isolated from ICU patients exhibited resistance to third-generation cephalosporins, and in 2003 this number was reported to have risen to ~20% (Gaynes and Edwards, 2005). This rapid increase is undoubtedly as a result of the evolution and dissemination of ESBLs amongst *K. pneumoniae* strains. Since the first identification of carbapenemases in the early 1980's, dozens of new enzymes and variants have been identified in Gram-negative organisms, particularly in the Enterobacteriaceae, exhibiting the rapid rate at which these enzymes can evolve (Queenan and Bush, 2007). There has been a marked increase in the frequency of *K. pneumoniae* UTIs between 1975 and 2003 where incidences were reported as 4.6 and 9.8% respectively, where the increase in antibiotic resistance in *K. pneumoniae* is undoubtedly a major contributing factor (Gaynes and Edwards, 2005). The existence of several of these resistance mechanisms in a single strain can render that strain MDR or even PDR, leaving clinicians in a quandary in deciding how to treat these infections. Such infections, not only *K. pneumoniae* but also those caused by other Gram-negative organisms including *A. baumannii* and *P. aeruginosa*, has led to an increased demand for the development of new antimicrobials and also the revival of the polymyxin, colistin (Falagas and Kasiakou, 2005).

The constant and fluid evolution of this bacterium in response to antibiotic exposure has resulted in the emergence of strains that are almost untreatable and consequently are capable of causing significant mortality. This bacterium has undoubtedly progressed and evolved to become a dangerous pathogen, one which we are struggling to combat and control in the clinical setting and which necessitates urgent attention. It is almost inevitable that for some species of bacteria we will witness a post-antibiotic era unless drastic action is taken.

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Appendix A

Abstracts of conference presentations

conducted during the tenure of this thesis

The Effect of Chlorpromazine on Multidrug Resistance Regulator RamA

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Background

RamA is an AraC/XylS family transcriptional activator found in *Klebsiella pneumoniae*, *Salmonella* spp. and *Enterobacter* spp., where its over expression has been shown to confer a multidrug resistant (MDR) phenotype via the upregulation of the efflux pump *acrAB*. In previous work, the phenothiazine, chlorpromazine (CPZ), has been shown to exhibit increased antimicrobial activity against ramA-deleted *Salmonella enterica* serovar Typhimurium. Thus the aim of this study was to characterise the antimicrobial properties of CPZ and its effect on the expression of *ramA* and its associated genes in *K. pneumoniae*.

Methods

A variety of clinical and environmental strains of *K. pneumoniae* which had been previously characterised as either *ramA* non or over expressers were used in this study. MIC and synergy testing was performed in the presence or absence of chlorpromazine according to the BSAC guidelines. RT-PCR was used to determine differences in gene transcription levels and these results were quantified using Bio-Rad Quantity One software.

Results

CPZ treatment increased the expression of *ramA*, *acrA*, *romA* and *tetR* in all strains tested. The ranges of fold expression increases were as follows: *ramA*, 1.6-6.4, *romA*, 1.9-4.1, *tetR* 0.8-2.3, and *acrA* 0-0.7. CPZ was found to act in synergy with certain antibiotics to result in up to 8-fold decreases in the MIC.

Conclusions

This study identified that CPZ acts as an inducer of *ramA* and its associated genes (*acrA*, *romA* and *tetR*). MIC testing showed that CPZ was able to act synergistically with certain antibiotics to reduce their respective MICs compared to those with the antibiotic alone.

RamR: A Dual Regulator of Antibiotic Susceptibility and Biofilm Formation in *Klebsiella pneumoniae* Kp342

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Objectives

RamA is an AraC/XylS family transcriptional activator found in *Klebsiella pneumoniae*, *Salmonella* spp. and *Enterobacter* spp., the over-expression of which is associated with an MDR phenotype. Recently a *tetR*-like gene that lies upstream of *ramA*, known as *ramR*, has been identified as a repressor of *ramA*. *K. pneumoniae* Kp342 is a diazotrophic endophyte strain which has been reported to exhibit notable resistance to antibiotics**.** Despite its MDR phenotype Kp342 has been shown in animals to be attenuated in comparison to clinical *K. pneumoniae* strains. The aims of this study were to: determine the levels of *ramA* expression and establish its role in Kp342's MDR phenotype; determine the effect of *ramR* complementation on *ramA* expression and antibiotic susceptibility.

Methods

Genome and sequence analysis performed in *K. pneumoniae* strain Kp342 demonstrated a 96bp deletion within the *ramR* gene. Cloning and complementation with full size wild type *ramR* was performed in Kp342 (hereby known as Kp342/ramR). RT-PCR was used to assess levels of gene expression which were subsequently quantified using Bio-Rad Quantity One software. MIC testing was performed against chloramphenicol (Cm), norfloxacin (Nor) and tetracycline (Tet) according to BSAC guidelines. Biofilm formation was measured using a modified protocol of O'Toole and Kolter.

Results

Kp342 containing the mutated *ramR* gene (96bp deletion) was shown to over-express *ramA* and the putative outer membrane protein *romA*. Complementation of the *ramR* gene resulted in the repression of both *ramA* and *romA* transcription by 3-4 fold. Interestingly, the *ramR* complemented strains were shown to have increased biofilm formation (up to 9-fold increase) over a 72 hour period in both LB and M9 medium after static growth at 37°C. MICs of the tested antibiotics were reduced up to 16-fold in Kp342/ramR compared to the *ramR* mutated Kp342.

Conclusions

This result demonstrates that *ramR* acts as a repressor of both *ramA* and putative outer membrane protein *romA* and as a result the bacterium becomes more susceptible to antibiotics. However the restoration of a functional *ramR* in Kp342 increases biofilm formation significantly, suggesting that *ramR* plays a role in the regulation of biofilm formation genes and possibly bacterial virulence.

The Differential Effect of Mutations in RamR, in Mediating Antibiotic Susceptibility in *Klebsiella pneumoniae*

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Objectives

The transcriptional activator RamA confers an antibiotic resistance phenotype in *Klebsiella pneumoniae* when over-expressed. Recently a *tetR*-like gene that lies upstream of *ramA*, known as *ramR*, has been identified as a repressor of *ramA*. Correspondingly clinical isolates of *K. pneumoniae* with mutations within *ramR* have been shown to over-express *ramA*; however *ramA* over-expresssing clinical isolates with no changes within the repressor *ramR* or within the associated promoter regions have also been found. Thus the aims of this study were: to firstly determine whether *ramA* over-expression mediated through *ramR*-derepression was dependent on the selective agent and secondly to determine whether mutations within the *ramR* gene would impact on resistance profiles upon complementation with wild-type *ramR*.

Methods

Laboratory mutants from *K. pneumoniae* Ecl8 were selected for by the culturing of exponential growth phase bacterial cultures on ciprofloxacin or chlorpromazine plates at four times the MIC. The *ramR* gene was amplified from a selection of the mutants and was sequenced. The subsequent MICs to chloramphenicol (Cm), norfloxacin (Nor) and tetracycline (Tet) of the selected mutants with changes within the *ramR* gene were then determined. *ramR* mutants were complemented with a plasmid containing the wild-type *ramR*, pACramR, and their subsequent MICs were determined to Cm, Nor and Tet as before.

Results

Four of the selected mutants were revealed to harbour mutations resulting in amino acid changes within the *ramR* gene. The mutations (G96D, S137Stop, E175K) found in the *ramR* gene appeared to favour the C-terminus region. The mutants exhibited 32-4 fold increases in MICs compared to the parental strain depending on which mutations were sustained within *ramR*. Complementation with the wild-type *ramR* resulted in 1-16 fold reductions in the MICs also dependent on the type of *ramR* mutations.

Conclusion

All the mutants appeared to sustain *ramR* changes regardless of the compound used in the selection, indicating that *ramR* is a critical factor in mediating *ramA* over-expression. The partial restoration of the parental phenotypes in the *ramR*-mutants indicates the MDR phenotypes are attributable to mutations within the RamR protein but another factor may be required to restore susceptibility to parental levels. The

mutations identified within RamR protein are clustered around the C-terminus suggesting the relative importance of this region in the derepression of *ramA*.

Mechanisms of Carbapenem Resistance Emerging During Therapy in a Strain of *Klebsiella pneumoniae* Treated With Meropenem

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Background

A clinical strain *Klebsiella pneumoniae* (K1), with reduced susceptibility to ertapenem, causing severe infection became highly carbapenem resistant (K2) during meropenem therapy.

Methods

MIC testing of K1 and K2 was performed by agar dilution. Strains were typed by PFGE with *XbaI*. Outer membrane proteins were extracted and separated by SDS PAGE. Mutation studies were performed with the carbapenems; ertapenem, imipenem and meropenem. PCR was used to amplify the *ompK35* and *ompK36* genes, which were sequenced. PCR was performed to detect the common β-lactamases and all known carbapenemases. RT-PCR was performed to assess expression of *ompK35*, *ompK36*, *ramA, romA* and *acrA*.

Results

Both strains were resistant to all antibiotics tested except only K2 was resistant to ertapenem and meropenem, with intermediate resistance to imipenem. Both strains contained the CTX-M-15 β-lactamase and were indistinguishable by PFGE. OMP profiling revealed both strains lacked an OMP of around 40kDa but K2 also lacked a major OMP of around 36kDa. Carbapenem mutation studies on K1 showed that the porin loss and resistance phenotype observed in K2 could easily be selected *in vitro* in K1. Carbapenem MICs performed with clavulanic acid showed inhibition of ertapenem resistance suggesting a contribution by the CTX-M-15 β-lactamase. None of the known carbapenemases were detected by PCR. RT-PCR showed the upregulation of *ramA*, *romA* and *acrA*, and no expression of *ompK35* as well as mutations in the OMPs of both strains.

Conclusions

PFGE showed K1 and K2 are isogenic, the main difference between them is porin loss which appears to have been selected for directly during meropenem therapy. It is likely that the carbapenem resistance phenotype is due to three contributing factors; the presence of CTX-M-15 and upregulation of efflux pumps to give reduced ertapenem susceptibility in K1 followed by porin loss, resulting in meropenem resistance in K2.

The Epidemiology of *Acinetobacter baumannii* of Animal Origin

Hamouda A, Findlay J, Al Hassan L, Amyes SGB
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Objectives

The aim of this study was to determine whether *A. baumannii* strains collected from animals slaughtered for human consumption from Scottish abattoirs possessed major epidemiological characteristics similar to strains isolated from clinical patients.

Methods

Sixteen *A. baumannii* isolates (8 from cattle and 8 from pigs) obtained from 1381 animal samples, collected between the period February 2006 to August 2007, were used in this study. For species identification, *bla*OXA-51-like genes were amplified by the polymerase chain reaction (PCR) and confirmed by sequencing. Minimum inhibitory concentration (MIC) of antibiotics was performed according to the BSAC guidelines. PCR was also used to amplify sections of DNA that harboured resistance islands (RIs), *bla*ampC and the insertion sequence *ISAba1*. All strains were genotyped by pulsed-field gel electrophoresis (PFGE) using *Xba*I restriction endonuclease.

Results

All isolates were confirmed as *A. baumannii* using *bla*OXA-51-like genes and, interestingly, many harboured the *bla*OXA-51 gene itself. The PFGE profile of the animal isolates showed some genotypic diversity and a very different profile from *A. baumannii* European clone I, II, and III. All isolates lacked any evidence of resistance islands and *ISAba1* but every strain harboured *bla*ampC genes. All 16 isolates were sensitive to imipenem (MIC ≤4mg/L), meropenem (MIC ≤4mg/L), ciprofloxacin (MIC ≤0.5mg/L) and piperacillin/tazobactam (MIC ≤16mg/L) but they were all (100%) resistant to ceftazidime (MIC >2mg/L). When animal isolates were compared with the European clones I, II, and III they belonged to a different species pool.

Conclusion

All isolates were confirmed as *A. baumannii* using *bla*OXA-51-like genes and, interestingly, many harboured the *bla*OXA-51 gene itself. The PFGE profile of the animal isolates showed some genotypic diversity and a very different profile from *A. baumannii* European clone I, II, and III. All isolates lacked any evidence of resistance islands and *ISAba1* but every strain harboured *bla*ampC genes. All 16 isolates were sensitive to imipenem (MIC ≤4mg/L), meropenem (MIC ≤4mg/L), ciprofloxacin (MIC ≤0.5mg/L) and piperacillin/tazobactam (MIC ≤16mg/L) but they were all (100%) resistant to ceftazidime (MIC >2mg/L). When animal isolates were

compared with the European clones I, II, and III they belonged to a different species pool.

Appendix B

Publications conducted during the tenure of this thesis

Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*

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Class D OXA-51-like carbapenemases are chromosomally encoded and appear to be intrinsic to *Acinetobacter baumannii* [1]. These enzymes are weak carbapenemases, and it has been suggested that they only confer carbapenem resistance if an additional promoter is provided by the insertion of IS*Aba1* upstream of the structural gene [2]. Following recent reports in *CMI* of new variants of the OXA-51-like subgroup of class D carbapenemases [3,4], we wish to report the identification of 11 more novel bla_{OXA-51}-like variants in clinical isolates of *A. baumannii*.

A collection of 60 clinical *A. baumannii* isolates of diverse worldwide origin was screened for bla_{OXA-51}-like genes using primers OXA-69A and OXA-69B, which amplify a 975-bp product containing the entire bla_{OXA-51}-like coding sequence [5]. Amplification products were sequenced on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK) and were compared using BLAST and Multalin software. Isolates producing a band of *c.* 2000 bp, as the result of an insertion upstream of the bla_{OXA-51}-like gene, were re-analysed using primers preABprom+ [6] and OXA-69B to produce a 1189-bp product, which was then sequenced as above.

Sequence comparison revealed 11 novel bla_{OXA-51}-like sequences, encoding 11 novel enzymes, designated OXA-79, OXA-80, OXA-82, OXA-104 and OXA-106–OXA-112 (http://www.lahey.org/studies/webt.asp). The origins of the isolates encoding these enzymes are listed in Table 1. These novel enzymes differ from all currently known enzymes by between one and four amino-acids (Table 1), sharing 99% amino-acid identity. The bla_{OXA-79}, bla_{OXA-80}, $bla_{OXA-106}$, $bla_{OXA-107}$, $bla_{OXA-109}$, $bla_{OXA-110}$ and $bla_{OXA-112}$ genes each differ from the gene encoding the closest related enzyme by a single nucleotide, resulting in a single amino-acid change. The nucleotide sequence of bla_{OXA-82} differs from bla_{OXA-66} by two nucleotides, one of which is silent ($T_{435} \rightarrow A$), and $bla_{OXA-111}$ has three silent substitutions compared to bla_{OXA-51} ($C_{90} \rightarrow T$, $T_{216} \rightarrow A$ and $T_{507} \rightarrow C$). The sequences of $bla_{OXA-104}$ and $bla_{OXA-108}$ differ from that of bla_{OXA-95} by 11 and five nucleotides, respectively, eight ($C_{54} \rightarrow T$, $C_{369} \rightarrow T$, $A_{390} \rightarrow G$, $G_{426} \rightarrow A$, $C_{549} \rightarrow T$, $C_{610} \rightarrow T$, $G_{720} \rightarrow A$ and $G_{732} \rightarrow A$) and one ($C_{328} \rightarrow T$) of which are silent. All amino-acid changes were located outside the class D carbapenemase motifs [7]. All isolates producing the larger *c.* 2000-bp product with primers OXA-69A and OXA-69B also yielded a 1189-bp product with the second set of primers, indicating that these isolates had an IS*Aba1* insertion upstream of the bla_{OXA-51}-like gene. Sequencing confirmed that the bla_{OXA-79}, bla_{OXA-80}, bla_{OXA-82} (in both isolates), $bla_{OXA-107}$ (in all isolates), $bla_{OXA-108}$, $bla_{OXA-109}$ and $bla_{OXA-110}$ (in both isolates) genes each had an IS*Aba1* insertion 7 bp upstream of the structural gene. A detailed analysis of the genotypic relationships among these isolates and the influence of the IS*Aba1* insertions on the expression of carbapenem resistance will be published separately.

Since the discovery of OXA-51 in 2004, the number of related enzymes in this group has now

Table 1. Amino-acid changes resulting in 11 novel enzymes

Related enzyme	Amino-acid change	Novel enzyme	Origin of isolates
OXA-66	$Trp_{222} \rightarrow Gly$ ($T_{664} \rightarrow G$)	OXA-79	UK
OXA-66	$Pro_{130} \rightarrow Leu$ ($C_{389} \rightarrow T$)	OXA-80	UK
OXA-66	$Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-82	Turkey, USA
OXA-95	$Phe_7 \rightarrow Leu$ ($C_{21} \rightarrow A$) $Asp_{147} \rightarrow Asn$ ($G_{440} \rightarrow A$) $Lys_{146} \rightarrow Asn$ ($G_{438} \rightarrow T$)	OXA-104[a]	USA
OXA-70	$His_{199} \rightarrow Asp$ ($C_{595} \rightarrow G$)	OXA-106	Estonia
OXA-69	$Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-107	Poland, Slovenia
OXA-95	$Phe_7 \rightarrow Leu$ ($C_{21} \rightarrow A$) $Ala_{215} \rightarrow Thr$ ($G_{73} \rightarrow A$) $Ala_{156} \rightarrow Thr$ ($G_{466} \rightarrow A$) $Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-108[a]	Poland
OXA-66	$Pro_{130} \rightarrow Gln$ ($C_{389} \rightarrow A$)	OXA-109	UK
OXA-69	$Ile_{139} \rightarrow Leu$ ($A_{415} \rightarrow C$)	OXA-110	Poland
OXA-51	$Thr_{24} \rightarrow Ser$ ($A_{70} \rightarrow T$) $Val_{86} \rightarrow Ala$ ($T_{143} \rightarrow C$) $Lys_{209} \rightarrow Met$ ($A_{626} \rightarrow T$)	OXA-111	Belgium
OXA-69	$Ser_{246} \rightarrow Pro$ ($T_{736} \rightarrow C$)	OXA-112	UK

[a]OXA-104 and OXA-108 are equally distant from OXA-65 and OXA-95, with Asn225 → Asp in place of Phe7 → Leu. Nucleotide changes are listed in parentheses.

increased to at least 39, and they appear to be ubiquitous in *A. baumannii* [3,5,7,8]. While the insertion of IS*Aba1* upstream of bla_{OXA-51}-like genes has been associated with resistance to carbapenems, not all isolates contain such an insertion [2]. OXA-51 and OXA-69 have been shown to hydrolyse carbapenems poorly [5,7], and it would be of interest to determine whether the sequence diversity of this group is a result of selection for greater activity against the carbapenems.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The novel sequences reported in this study are deposited in the GenBank nucleotide database under accession numbers EU019534 (OXA-79), EU019535 (OXA-80), EU019536 (OXA-82), EF581285 (OXA-104), EF650032 (OXA-106), EF650033 (OXA-107), EF650034 (OXA-108), EF650035 (OXA-109), EF650036 (OXA-110), EF650037 (OXA-111) and EF650038 (OXA-112).

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Mumps vaccine failure or vaccination scheme failure?

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We read with interest the study by Atrasheuskaya *et al.* [1] concerning vaccine failure in cases of mumps during 2002–2004 in Novosibirsk, Russia. According to Atrasheuskaya *et al.* [1], the question of whether these cases were caused by an absence of sufficient titres of antibody in response to vaccination, or were caused by the absence of neutralising antibody specific for the causative mumps virus strain, cannot be answered conclusively. On the basis of lessons learnt from a descriptive epidemiological investigation of a mumps outbreak in 214 young adults in Austria during 2006, we hypothesise that susceptibility to mumps infection is less likely to result from a vaccine failure than from a vaccination scheme failure, i.e., administration of only one of the two recommended doses of vaccine.

Active immunisation with the Jeryl Lynn strain of attenuated mumps virus vaccine has been available in Austria since 1974, when a bivalent mumps–measles (MM II) vaccine produced by Merck Sharp & Dohme was introduced as part of the national childhood immunisation programme. In 1994, the bivalent vaccine was replaced by a trivalent mumps, measles and rubella (MMR) vaccine produced by Pasteur Merieux Connaught (containing the Jeryl Lynn strain). Since 2001, the Priorix MMR vaccine (Glaxo Smith Kline) has been in use, which contains the RIT 4385 mumps strain derived from the Jeryl Lynn strain. The vaccination regimen includes two doses, with the first dose

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Effect of frameshift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*

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Acinetobacter baumannii is a Gram-negative pathogenic bacterium that often exhibits a multidrug-resistant phenotype causing infections at various sites of the body and increasingly leading to septicaemic shock. This study evaluated the role of acriflavine, a frameshift mutagen, on the movement of insertion sequence IS*Aba1* in clinical isolates of *A. baumannii*, with the focus on changes in expression levels of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes. Resistance profiles were assessed with consideration of IS*Aba1* acting as a promoter upstream of the bla_{ADC} or $bla_{OXA\text{-}51\text{-}like}$ gene. IS*Aba1* movement was observed in the acriflavine mutants Ab153M and Ab1225M. Ab153M exhibited an increase in the MIC values of carbapenems and ceftazidime, with IS*Aba1* gained upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes, correlating with an increase in gene expression. Reduced expression of the 17, 23 and 25 kDa outer-membrane proteins (OMPs) was also observed in Ab153M. There was a significant decrease in MIC values of carbapenems with the loss of IS*Aba1* upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes in strain Ab1225M, and a significant decrease in $bla_{OXA\text{-}51\text{-}like}$ gene expression and, to a lesser extent, in bla_{ADC} expression. Ab1225M and a serially subcultured Ab1225 strain (Ab1225s) exhibited overexpression of the 17, 23, 25 and 27 kDa OMPs. There was a decrease in MIC values of the carbapenems and piperacillin/tazobactam but not of ceftazidime in Ab1225s, which had IS*Aba1* upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes. A significant decrease in $bla_{OXA\text{-}51\text{-}like}$ expression was observed in Ab1225s, whereas the expression of bla_{ADC} was similar to that in the Ab1225 parental strain. The attenuation in this strain may be due to overexpression of OMPs and it is clear that, even if IS*Aba1* is present upstream of an antibiotic resistance gene, it may not necessarily contribute towards the overexpression of antibiotic resistance genes ($bla_{OXA\text{-}51\text{-}like}$ in Ab1225s). Movement of the IS element within the *A. baumannii* chromosome may be an important regulatory mechanism employed by the bacterium under particular stress conditions, and the ability to upregulate the expression of antibiotic resistance genes is likely to be an important factor in the pathogenicity of this bacterium.

INTRODUCTION

Acinetobacter baumannii is one of the most difficult noscocomial pathogens to treat, similar to other bacterial pathogens such as meticillin-resistant *Staphylococcus aureus*, *Clostridium difficile* and *Pseudomonas aeruginosa*. Hospital-acquired pneumonia is the most common infection caused by this organism (Peleg *et al.*, 2008).

Nosocomial infections may be transmitted via aerosols, staff or even by inadequately disinfected medical devices (Spelman, 2002). Other common infections caused by this bacterium are ventilator-associated pneumonia, urinary tract infections and bacteraemia (Perez *et al.*, 2007). It can also cause suppurative infections of the skin, and abdominal and central nervous system infections (Fournier & Richet, 2006). The bacterium has developed resistance to different types of β-lactam antibiotics by the production of β-lactamases, which include plasmid-encoded class A (TEM/PER-1/VEB/SHV/CTX-M), class B (metallo-β-lactamases), class C (*Acinetobacter* derived cephalosporinases AmpC/bla_{ADC}) and class D (the oxacillinases: OXA-23-like, OXA-40-like, OXA-58-like and OXA-51-like, the latter being an intrinsic β-lactamase of *A. baumannii*) β-lactamases (Brown & Amyes, 2006; Perez *et al.*, 2007).

Outer membranes, like other biological membranes, are built as lipid bilayers that are permeable to hydrophobic molecules and are involved in the influx of nutrients and the efflux of toxic substrates or antibiotics which can hamper normal cell function (Nikaido, 2003). Three outer-membrane proteins (OMPs) have been reported to be missing in the imipenem-resistant strains of *A. baumannii*: the first is a 33–36 kDa protein, the second is a 29 kDa protein designated CarO and the last is a 43 kDa protein

Abbreviation: OMP, outer-membrane protein.

that shows significant peptide homology with OprD from *P. aeruginosa* (Vila *et al.*, 2007).

Transposable elements are important in providing genetic variability and are generally tightly regulated (Mugnier *et al.*, 2009). Insertion sequences are capable of independent transposition in the microbial genome and are also responsible for the spread of resistance and virulence determinants within the species (Mugnier *et al.*, 2009). IS*Aba1* has been associated with the expression of several antibiotic resistance genes including $bla_{\text{OXA-51-like}}$ and bla_{ADC} (Corvec *et al.*, 2003, 2007). It is also known that frameshift products are involved in transposition of IS*629*, a member of the IS*3* family (Chen & Hu, 2006; Mahillon & Chandler, 1998). IS*Aba1* belongs to the IS*4* family, and it has been reported recently that it is capable of transposition and that the transposase gene is downregulated by translational frameshifting (Mugnier *et al.*, 2009). It has also been reported that IS*4Bsu1*, a member of the IS*4* family in *Bacillus subtilis*, is responsible for the genetic instability of poly-γ-glutamic acid production and that IS*1999*, a member of the same family, is responsible for expression of β-lactam resistance genes (Aubert *et al.*, 2006; Nagai *et al.*, 2000).

Acriflavine is known to cause frameshift mutations by intercalation in DNA (Kornberg, 1980). In this study, we evaluated the role of OMPs, other than the major OMPs, that might be involved in the transport of nutrients and influx of antibiotics, particularly imipenem, meropenem, ceftazidime and piperacillin/tazobactam, by comparing a parental strain, an acriflavine-resistant mutant and a strain serially subcultured on MacConkey agar. The role of acriflavine as a frameshift mutagen was evaluated with respect to its effect on the control of $bla_{\text{OXA-51-like}}$ and bla_{ADC} as a result of the transposition of IS*Aba1*. This study also aimed to identify the changes in levels of expression of antibiotic resistance genes due to the movement of IS*Aba1*.

METHODS

The bacterial strains used this study were *A. baumannii* 153 (Ab153) (Nottingham, UK), *A. baumannii* 153 acriflavine mutant (Ab153M), *A. baumannii* 1225 (Ab1225) (Wroclaw, Poland), *A. baumannii* 1225 acriflavine mutant (Ab1225M) and *A. baumannii* 1225 serially subcultured strain (Ab1225s) (Wroclaw, Poland). All parental strains were kindly supplied by Dr K. J. Towner (Queens Medical Centre, Nottingham, UK).

Identification and typing. Isolates were screened for the conserved 16S–23S rRNA intergenic spacer sequence using primer 1 (5′-TTGTACACACCGCCCGTCA-3′) and primer 2 (5′-GGTACTTAGATGTTTCAGTTC-3′). The expected product size was 975 bp. Restriction of the product by the enzyme *Alu*I produced fragments of 50, 125, 135, 165, 175 and 330 bp specific for *A. baumannii*. Restriction of the same product with *Nde*II produced fragments of 50, 110, 145, 330 and 360 bp specific for *A. baumannii* (Dolzani *et al.*, 1995).

The strains were further identified as *A. baumannii* by amplification of the intrinsic $bla_{\text{OXA-51-like}}$ gene by PCR with the primers oxa-69A (5′-CTAATAATTGATCTACTCAAG-3′) and oxa-69B (5′-CCAGTGGATGGATAGATTATC-3′) at an annealing temperature of 48 °C. The expected product sizes were 975 bp for strains without IS*Aba1* present upstream of the $bla_{\text{OXA-51-like}}$ gene and 2155 bp for strains with IS*Aba1* present upstream of the $bla_{\text{OXA-51-like}}$ gene (Héritier *et al.*, 2005).

The strains were screened for the bla_{ADC} gene with primers ISADC1 (5′-GTTGCACTTGGTCGAATGAAAA-3′) and ISADC2 (5′-ACGTCGCGAGTTGAAGTAAGTT-3′) with an annealing temperature of 51 °C. The expected product size was 751 bp if IS*Aba1* was located upstream of the bla_{ADC} gene (Ruiz *et al.*, 2007).

Mutational analysis and strain subculture. Strains Ab153 and Ab1225 were subjected to treatment with acriflavine after overnight growth in nutrient broth. Mutants were isolated using a gradient plate technique as described previously with a concentration range between 0.03 and 0.3 % (Hunt & Sandham, 1969). A single colony at the highest concentration was selected and tested for further analysis. Strain Ab1225s was serially subcultured daily on MacConkey agar for more than 150 days in order to check for OMP changes and the stability of IS*Aba1*.

Antimicrobial susceptibility testing. The isolates were tested for their susceptibility to imipenem, meropenem, ceftazidime and piperacillin/tazobactam. MICs were determined by the agar double-dilution method according to British Society for Antimicrobial Chemotherapy (BSAC) methodology (Anonymous, 1991). The results were interpreted according to BSAC guidelines. The reference strains used for MIC testing were *A. baumannii* ATCC 19606, *Escherichia coli* NCTC 10418, *P. aeruginosa* NCTC 10662 and *S. aureus* NCTC 6571 (Andrews, 2007).

Analysis of gene expression. Expression of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes was analysed by RT-PCR. The primers used for bla_{ADC} were ADC1 (5′-CCGCGACAGCAGGTGGATA-3′) and ADC2 (5′-TCGGCTGATTTTCTTGGTT-3′) with an annealing temperature of 51 °C, producing a product of 451 bp (Ruiz *et al.*, 2007). The primers used for $bla_{\text{OXA-51-like}}$ were 51F (5′-TTTCAGCCTGCTCACCTT-3′) and 51R (5′-TTCCCTTGAGGCTGAACAAC-3′) with an annealing temperature of 56 °C, producing a product of 679 bp (this study). Total RNA was extracted from isolates in the exponential growth phase using a RiboPure Bacteria kit (Ambion) and treated with the DNase I provided in the kit. cDNA was synthesized from 250 ng RNA using an Access Quick RT-PCR System kit (Promega). PCR products were run on an agarose gel and stained with GelRed (Cambridge Bioscience) for visualization. PCR products were quantified using Quantity One Software version 4.6.1 (Bio-Rad). The 16S rRNA gene was amplified as an internal control using primers 16S-F (5′-GACGTACTCGCAGAATAAGC-3′) and 16S-R (5′-TTAGTCTTGCGACCGTACTC-3′) at an annealing temperature of 56 °C (Lin *et al.*, 2009). The product size was 426 bp.

Analysis of OMP profiles. Strains were grown overnight in nutrient broth and OMP extraction was performed using a method described previously (Bossi & Bossi-Figueroa, 2007). The protein profiles were studied by 10 % SDS-PAGE with Molecular Weight Standards, Broad Range (New England Biolabs) used as a size marker. The protein concentration was estimated after reconstitution in buffer and approximately 45 µg protein was loaded into each well. After electrophoresis, the gels were stained with Coomassie Blue R-250 (Sigma).

RESULTS

Identification and typing

The isolates were screened for the conserved 16S–23S rRNA intergenic spacer sequences using primer 1 and

primer 2 as described above, amplifying a product of 975 bp, specific to *A baumannii*.

A fragment of approximately 2155 bp was obtained for strains Ab1225 and Ab1225s after amplification with the oxa-69A and oxa-69B primers, suggesting that the IS*Aba1* element was present upstream of the $bla_{\text{OXA-51-like}}$ gene. If the IS*Aba1* element was not present upstream, the fragment size was 975 bp, which was the size obtained for strain Ab153. IS*Aba1* was found upstream of the bla_{ADC} gene in strains Ab1225 and Ab1225s, giving a product of 751 bp. To check for overexpression of the $bla_{\text{OXA-51-like}}$ gene and transposition of the IS*Aba1* element, mutational analysis was performed using the dye acriflavine as a frameshift mutagen.

Sequencing of the $bla_{\text{OXA-51-like}}$ gene revealed that $bla_{\text{OXA-66}}$ was present in strain Ab153 and $bla_{\text{OXA-110}}$ in strain Ab1225.

Mutational analysis

IS*Aba1* movement was recorded in Ab1225M and Ab153M after acriflavine treatment had been performed. It was observed that strain Ab1225M lost its IS*Aba1* element, previously present upstream of the $bla_{\text{OXA-51-like}}$ gene, giving a product size of 975 bp, whereas strain Ab153M gained the IS*Aba1* element upstream of the $bla_{\text{OXA-51-like}}$ gene with a product size of 2155 bp.

IS*Aba1* movement was seen upstream of the bla_{ADC} gene in the acriflavine-treated Ab153M strain, but its loss was observed in the similarly treated strain Ab1225M. IS*Aba1* movement was not seen in the subcultured strain Ab1225s and its features remained identical with respect to the parental strain Ab1225. The IS*Aba1* sequence of strains Ab153M, parental Ab1225 and Ab1225s did not show any mutations or nucleotide substitutions.

Antimicrobial susceptibility testing

Table 1 shows the changes in MICs in the *A. baumannii* parental, mutant and serially subcultured strains. An eightfold rise in MICs for imipenem was seen in Ab153M and an eightfold decrease was seen in Ab1225M with respect to the parental strains Ab153 and Ab1225. A fourfold and twofold decrease in MIC for imipenem and meropenem was seen in Ab1225s with respect to Ab1225. There was negligible difference seen in MIC values for ceftazidime. No major difference was seen in the MIC values of piperacillin/tazobactam except for a fourfold decrease observed in strain Ab1225s in comparison with the Ab1225 parental strain. As no IS*Aba1* change was observed in Ab1225s, it was important to see whether there were any significant changes related to the OMPs of this strain, as it had a fourfold and twofold decrease in MIC for imipenem and meropenem compared with its parent, Ab1225.

Analysis of gene expression

Expression of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes was analysed by RT-PCR and the products obtained were quantified using Quantity One software version 4.6.1. Product analysis determined that there was 2.7-fold increase in expression of the bla_{ADC} gene for Ab153M compared with Ab153. There was a small decrease (1.2-fold) seen in expression of the bla_{ADC} gene for Ab1225M compared with Ab1225. A negligible difference was seen in expression of the bla_{ADC} gene between Ab1225 and Ab1225s. It was observed that there was a 2.5-fold increase in expression of the $bla_{\text{OXA-51-like}}$ gene in Ab153M compared with Ab153. There was a fivefold decrease in expression of $bla_{\text{OXA-51-like}}$ in Ab1225M compared with Ab1225, and a fourfold decrease in expression of $bla_{\text{OXA-51-like}}$ in Ab1225s compared with Ab1225. This was confirmed three times and the results recorded were based on the mean increase or decrease of individual strains.

Analysis of OMP profiles

Fig. 1 shows the OMP profiles obtained for the *A. baumannii* parental, mutant and serially subcultured strains. Analysis of the OMP profiles revealed significant differences between the Ab153 parental strain and Ab153M. There was reduced expression of the 17, 23 and 25 kDa OMPs in the Ab153M strain with an increase in

Table 1. MICs of various antibiotics in the *A. baumannii* strains

Strain	MIC (mg l^{-1})				Expression of $bla_{\text{OXA-51-like}}$ gene*	Expression of bla_{ADC} gene*
	Imipenem	Meropenem	Piperacillin/tazobactam	Ceftazidime		
Ab153	0.25	1	32	32	0	0
Ab153M	2	4	64	128	+2.5	+2.7
Ab1225	2	4	64	128	0	0
Ab1225M	0.25	1	64	64	−5	−1.2
Ab1225s	0.5	2	16	128	−4	0

*Fold increase/decrease.

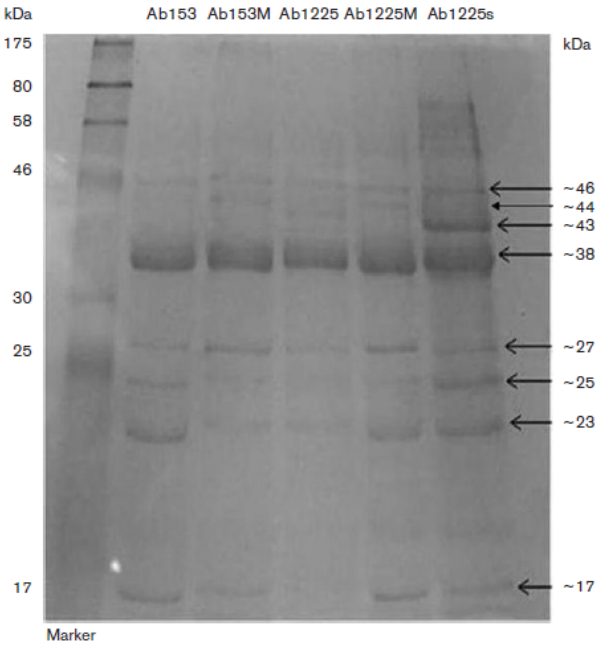

Fig. 1. OMP profiles of *A. baumannii* isolates. Ab153, parental strain; Ab153M, acriflavine mutant strain; Ab1225, parental strain; Ab1225M, acriflavine mutant strain; Ab1225s, MacConkey agar-subcultured strain.

MIC values. Ab1225M and Ab1225s exhibited an increase in expression of the 17, 23, 25 and 27 kDa OMPs with a decrease in MICs, rendering the strain more susceptible to some antibiotics. The OMP pattern produced by Ab1225s showed significant overexpression of a 43 kDa OMP.

DISCUSSION

All the *A. baumannii* strains were shown to exhibit different resistance profiles, which correlated with the movement of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. The MIC values for the Ab153 and Ab1225 parental and mutant strains suggested that IS*Aba1* may act as a promoter for the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. This is dependent upon IS*Aba1* acting as a promoter rather than just being present upstream of the antibiotic resistance gene. This also addressed the fact that IS*Aba1* may have a preferential role with respect to specific antibiotic resistance genes.

Strain Ab1225s had negligible effect on expression of the bla_{ADC} gene with respect to its Ab1225 parent, both of which had IS*Aba1* present upstream of the bla_{ADC} gene. This is supported by the fact that resistance to oxyiminocephalosporins is mainly due to hyperproduction of AmpC-type β-lactamases (Rodríguez-Martínez *et al.*, 2010). Ab1225s showed a fourfold decrease in $bla_{\text{OXA-51-like}}$ gene expression concurring with MICs to the carbapenems. There was increased expression of the 17, 23, 25, 27 and 43 kDa OMPs in Ab1225s, which led to a fourfold decrease in the MIC for imipenem, a twofold decrease in the MIC for meropenem and fourfold drop in the MIC for piperacillin/tazobactam. It was concluded that OMPs play a role in antibiotic resistance depending on the stress load of the compound and that they might be important for transport of the compound across the cell wall in either direction. The attenuation in Ab1225 may be due to OMP overexpression.

It was observed that all the *A. baumannii* isolates were resistant to ceftazidime, a fourth-generation cephalosporin, and thus IS*Aba1* may play a preferential role in increased expression of the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. This concurs with the fact that genetic organization of genes and their control play a crucial role in antibiotic resistance.

It was also observed that there was overexpression of OMPs in Ab1225M, which correlated with a decrease in MIC values for different antibiotics. Strains Ab153 and Ab153M had changes with respect to their OMP profiles. As an eightfold increase in resistance to imipenem and fourfold increase to meropenem was seen in Ab153M, it can be deduced that the resistance developed in Ab153M may be due not only to the movement of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene but also to a contribution by the reduced expression of three OMPs corresponding to the 17, 23 and 25 kDa proteins, all of which were poorly expressed in the mutant strain.

In contrast, overexpression of the 17, 23, 25 and 27 kDa OMPs could be seen in Ab1225M with a decrease in MIC

values, suggesting that these proteins may have some implications with respect to the increased susceptibility in this strain, which had lost IS*Aba1* upstream of the $bla_{OXA\text{-}51\text{-}like}$ and bla_{ADC} genes. The MICs of ceftazidime and piperacillin/tazobactam in Ab153 and Ab1225 parent and mutant strains coupled with the changes in β-lactamase expression may indicate the contributions made by the individual enzymes to the level of resistance; for example, changes in expression of the bla_{ADC} gene mirrored changes in the level of ceftazidime resistance.

It is clear from this study that the movement of IS*Aba1* is a strong factor for conferring resistance provided it is crucial for the gene action; if there is overexpression of primary surface structures of the bacterium, it can render the organism sensitive to a particular drug or poison.

Switching on the gene may be advantageous to this bacterium when it is required, as OMP changes can act synergistically; this was seen in Ab1225s and other strains and this depends on the action of promoters present for specific genes. This undoubtedly contributes to the organism's plasticity, making it resistant to different classes of antibiotics. Further studies to decipher the role of various factors involved in the pathogenesis of *A. baumannii* are necessary in order to understand its emergence as a multidrug-resistant pathogen.

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Rapid acquisition of decreased carbapenem susceptibility in a strain of *Klebsiella pneumoniae* arising during meropenem therapy

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Abstract

A strain of *Klebsiella pneumoniae* (K1) was isolated from a catheterized patient with a urinary tract infection. The patient was subsequently treated with meropenem, after which *K. pneumoniae* (K2) was once again isolated from the patient's urine. Susceptibility testing showed that strain K1 was fully susceptible to carbapenem antibiotics with the exception of ertapenem, to which it exhibited intermediate resistance, whilst K2 was resistant to ertapenem and meropenem. From pulsed-field gel electrophoresis profiling both strains exhibited identical banding patterns. Both contained CTX-M-15, OXA-1, SHV-1 and TEM-1 β-lactamase genes following PCR analyses. Outer membrane protein analysis demonstrated that K1 and K2 lacked an OMP of *c.* 40 kDa, with an additional OMP of *c.* 36 kDa missing from K2. Mutation studies showed that the K2 OMP phenotype could be selected by single-step carbapenem-resistant mutants of K1. Expression of transcriptional activator *ramA* and efflux pump component gene *acrA* were up-regulated in both strains by RT-PCR. Neither strain expressed *ompK35*, but *ompK36* was found in both. Sequence analysis revealed gene sequences of *ompK35*, *ompK36* and *ramR* in both strains; notably, *ramR* contained a mutation resulting in a premature stop codon. Transconjugation studies demonstrated transfer of a plasmid into *E. coli* encoding the CTX-M-15, TEM-1 and OXA-1 β-lactamases. We concluded that the carbapenem-resistant phenotype observed from this patient was attributable to a combination of CTX-M-15 β-lactamase, up-regulated efflux and altered outer membrane permeability, and that K2 arose from K1 as a direct result of meropenem therapy.

Keywords: CTX-M-15, efflux, ertapenem, *Klebsiella pneumoniae*, outer membrane proteins
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Introduction

Over the last three decades an increasing number of antibiotic resistance mechanisms have been emerging in members of the Enterobacteriaceae, particularly to the β-lactam antibiotics [1,2]. Resistance to these agents is most commonly mediated by either the production of enzymes, β-lactamases, which act to hydrolyse and therefore disable the antibiotics, or by the alteration of the expression of the outer membrane proteins (OMPs), which can act to decrease the diffusion of antibiotics through the cell membrane, preventing access to their target site [3,4]. Often a combination of both can result in extremely high levels of resistance to most, if not all, β-lactam antibiotics [3]. The loss of expression of two of the major OMPs in *K. pneumoniae*, OmpK35 and OmpK36, has been shown to contribute significantly to reduced susceptibility to β-lactam antibiotics in several studies [4,5]. Studies suggest that such porin loss coupled with extended-spectrum β-lactamase (ESBL) production can confer resistance to β-lactam antibiotics, including carbapenems [5,6].

It has previously been shown that reduced susceptibility to both ertapenem and meropenem can be achieved via the overexpression of efflux genes, in particular, members of the resistance-nodulation division (RND) family; however, imipenem susceptibility remains unchanged, suggesting that it is

not a good substrate for these efflux systems [7–9]. RND family efflux pumps have been shown to have a broad substrate range, demonstrating that the activation of a single resistance mechanism can result in clinical levels of resistance to several classes of antibiotics [10]. Overexpression of efflux pumps has been shown to contribute to carbapenem resistance in *Pseudomonas aeruginosa*, usually in conjunction with porin loss and/or *ampC* overexpression [7,11].

Klebsiella pneumoniae is known to cause a variety of serious nosocomial infections, particularly in immunocompromized patients. In recent years, the incidence of carbapenem resistance in *K. pneumoniae* has increased significantly, largely due to the production and dissemination of carbapenemases such as the KPC enzymes [12]. A study by the National Healthcare Safety Network in the USA over a 12-month period from 2006 to 2007 revealed that *K. pneumoniae* comprised 5.8% of the total hospital-acquired infections (HAIs) and 7.7% of catheter-associated urinary tract infections (UTIs) [13]. The same study showed that 21.2% of those *K. pneumoniae* isolates were resistant to cephalosporins and 10.1% exhibited resistance to carbapenems [13]. These drugs are traditionally viewed as the 'drugs of last resort' for multidrug-resistant (MDR) *K. pneumoniae* infections.

In this report we describe the *in vivo* clinical development of carbapenem resistance in a strain of *K. pneumoniae* after meropenem therapy.

Materials and Methods

Bacterial strains

K. pneumoniae strains K1 and K2 were found in separate urine samples from a catheterized patient with a UTI, in Hairmyres Hospital, Scotland, in 2008. K1 was collected prior to meropenem therapy and K2 was collected after meropenem therapy 5 days later. Carbapenem-susceptible *K. pneumoniae* ATCC 13883 was used as a control strain for OMP profiling and susceptibility testing. *K. pneumoniae* MGH78578, a clinical strain that contains the β-lactamases TEM-1 and SHV-12 and does not produce the OMP OmpK35 [14,15], was used as a control strain in mutation studies. Two previously characterized CTX-M-15 β-lactamase-containing *K. pneumoniae* strains, CTX-M-15 (1) and CTX-M-15 (2) (46th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract no. 86), were included as controls for susceptibility disc testing. A KPC-2-containing *K. pneumoniae* was used as a control for the modified Hodge test and *Escherichia coli* 25922 was used as the indicator strain. Rifampicin-resistant *E. coli* J62-2 was used as a recipient strain in transconjugation studies.

Susceptibility testing

Susceptibility testing was performed by agar doubling dilution according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines [16]. MICs were determined to the following antibiotics: amikacin, ampicillin, aztreonam, cefoxitin, ceftazidime, chloramphenicol, ciprofloxacin, colistin, ertapenem, gentamicin, imipenem, imipenem/EDTA, meropenem, piperacillin, sulbactam, tetracycline, tigecycline and tobramycin. Clavulanic acid synergy testing was performed with clavulanic acid incorporated into Iso-sensitest (IST) agar (Oxoid, Hampshire, UK) at 4 mg/L and using 10 μg ertapenem discs (Oxoid).

Pulsed-field gel electrophoresis (PFGE) typing

PFGE was performed according to the protocol of Miranda *et al.* [17]. Plugs were digested with *Xba*I. PFGE was performed with a CHEF-DRII system (Bio-Rad, Hertfordshire, UK) with a pulse time of 5–40 s over 22 h at 200 V. A Lambda Ladder PFG Marker (New England Biolabs, Hertfordshire, UK) was used as a size standard.

PCR detection of β-lactamase genes

Isolates K1 and K2 were screened by PCR for the presence of the genes for the following β-lactamases: AmpC, CTX-M, GES, GIM, IMP, KPC, NDM, NMC/IMI, OXA, PER, SHV, SIM, SME, SPM, TEM, VEB and VIM. Positive control clinically-isolated strains were used for each PCR. Amplification was carried out using cell lysate with Promega Go-*Taq* polymerase (Promega, Hampshire, UK) and all products were sequenced. All primers used in this study are listed in Table 1.

Modified Hodge test

A modified Hodge test was performed to detect carbapenemase activity in strains K1 and K2 [18].

Analysis of gene expression

Total RNA was extracted from isolate cultures in the exponential growth phase (OD $\sim$ 0.5) using the RiboPure Bacteria kit (Ambion, Warrington, UK) and treated with DNase I. cDNA was synthesized from 120 ng of RNA using the ProtoScript M-MuLV First Strand cDNA Synthesis kit (New England Biolabs). One microlitre of cDNA was used in PCR reactions. PCR reactions were run on an agarose gel and stained with ethidium bromide for visualization. All assays were performed in duplicate and with genomic DNA controls.

Outer membrane profiling

Outer membrane proteins were extracted according to the protocol of Tsu-Lan *et al.* [23]. Cultures were grown in both high osmolarity medium (LB broth) and low osmolarity medium (NB broth) as OmpK35 expression is known to be

TABLE 1. The primers used in this study

Primers	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product size (bp)	References
Gene detection and sequencing				
AmpC_F & R	ATCAAAACTGGCAGCCG	GAGCCCGTTTTATGCACCCA	550	[6]
CTX_F & R	GACGTCCGTATTTGCCTTC	ACCGTCGGTGACGATTTTAG	985	[19]
GES_F & R	ATGCGCTTCATTGACGCAC	CTATTTGTCCGTGCTCAGG	864	[20]
GIM_F & R	TCGACACACCTTGGTCTGAA	AACTTCCAACTTTGCCATGC	477	[21]
IMP_F & R	GGAATAGAGTGGCTTAAYTCTC	CCAAACYACTASGTTATCT	188	[21]
KPC_F & R	CAGCTCATTCAAGGGCTTTC	GGCGGCGTTATCACTGTATT	196	This work
NDM_F & R	GACCGATGACCGCCCAG	GACTTGGCCTTGCTGTCCTT	372	This work
NMC/IMI_F & R	ATGTCATTAGGTGATATGGC	GCATAATCATTTGCCGTACC	398	[6]
OXA_F & R	CCGTTAAAATTAAGCCCTTT	GGTCTGTGACTTTGCCGTCT	994	This work
PER_F & R	ATGAATGTCATTATAAAAGC	AATTTGGGCTTAGGGCAGAA	925	[20]
SHV_F & R	CGCCGGGTTATTCTTATTTG	CCACGTTTATGGCGTTACCT	1069	This work
SIM_F & R	TACAAGGGATTCGGCATCG	TAATGGCCTGTTCCCATGTG	570	[21]
SME_F & R	TAGAGGAAGACTTTGATGGG	GCATAATCATTCGCAGTACC	636	[6]
SPM_F & R	AAAATCTGGGTACGCAAACG	ACATTATCCGCTGGAACAGG	271	[21]
TEM_F & R	AGACGTCAGGTGGCACTTTT	GGCACCTATCTCAGCGATCT	977	[22]
VEB_F & R	CGACTTCCATTTCCCGATGC	GGACTCTGCAACAAATACGC	643	[20]
VIM_F & R	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	390	[21]
OmpK35_F & R	ATTTTGCACAAAAGGGGATG	AGAATTGGTAAACGATACCCACG	1117	This work & [6]
OmpK36_F & R	CAGCACAATGAATATAGCCGAC	GCTGTTGTCGTCCAGCAGGTTG	1126	[6]
RamR_F & R	CATCCCGGAGGCTTTATGAT	CGCTCGACCTTAAACACGTC	862	This work
RT-PCR analysis				
ramA_RTF & RTR	CTGCAACGGCTGTTTTTACA	GTGGTTCTCTTTGCGGTAGG	334	This work
acrA_RTF & RTR	GTCCTCAGGTCAGTGGCATT	GGTGCCCAACAGTTTCTGAT	200	This work
ompK35_RTF & RTR	AAAACGGCAACAAACTGGAC	AGACGGGTTTTTGTGGTCTG	211	This work
ompK36_RTF & RTR	GCGCTCTGTCTCCTACCAAC	GGTGTACTGAGTGGCCAGGT	188	This work
16S_RTF & RTR	CAGCCACACTGGAACTGAGA	GTTAGCCGGTGCTTCTTCTG	220	This work

inhibited in the former [24]. Protein concentrations were estimated using a Nanodrop 1000 spectrophotometer and *c.* 50 μg was separated on a 12% SDS PAGE gel. Gels were stained with coomassie blue.

Mutation studies

Overnight broth cultures of K1 and MGH78578 were spread onto IST agar plates containing either ertapenem or meropenem at 4–16× their respective MICs. Plates were incubated for 24 h and colonies were picked for further analysis of their susceptibility and OMP profiles.

Sequencing of *ompK35*, *ompK36* and *ramR*

OMP genes *ompK35* and *ompK36* and *ramR* were amplified and sequenced from strains K1, K2 and ATCC 13883. OMP gene sequences of ATCC 13883 and the *ramR* gene sequence from the sequenced genome of *K. pneumoniae* MGH78578 (NC 009648) were used as comparators.

Plasmid profiling

The plasmids of K1 and K2 were profiled using the S1 nuclease/PFGE method [25]. Briefly, agarose plugs of K1 and K2 were digested at 37°C for 45 min with 8 U of S1 nuclease. Reactions were stopped with the addition of 0.5 M EDTA and plugs were run by PFGE under the same conditions as used for *Xba*I typing.

Transconjugation studies

Transconjugation assays were performed as follows: overnight cultures of the recipient (*E. coli* J62-2) and donor strains (K1 and K2) were mixed in a 1:4 ratio, and cells were collected by centrifugation and resuspended in 30 μL of cold saline. Five microlitre aliquots of the resuspension were spotted onto a nutrient agar plate and incubated at 37°C for 6 h. Growth was collected and resuspended in cold saline and inoculated onto nutrient agar plates containing rifampicin at 16 mg/L and one of the selective antibiotics, gentamicin, ceftazidime or meropenem, at a range of concentrations.

Results

Susceptibility profiles

Both strains K1 and K2 were resistant to most antibiotics tested, with the following exception: K2 was resistant to both ertapenem and meropenem, with intermediate resistance to imipenem, whilst K1 was susceptible to all three (Table 2). Colistin was the only antibiotic to which both strains were susceptible. Antibiotic disc testing in the presence of clavulanic acid showed that ertapenem disc diameters increased significantly in the presence of clavulanic acid for strains K1, K2, CTX-M-15 (1) and CTX-M-15 (2) but no change was observed for ATCC 13883 or MGH78578 (Table 3).

PFGE analysis

Strains K1 and K2 showed identical banding patterns by PFGE analysis, indicating that they are of the same clonal type (Fig. 1). This suggests that K1 and K2 are almost certainly isogenic, K1 being the progenitor of K2.

TABLE 2. The susceptibility profiles of K1, K2, the K1 meropenem mutant, the K1 ertapenem mutant, MGH78578 and the MGH78578 meropenem mutant

Antibiotic	K1	S/I/R	K2	S/I/R	K1_Mero	S/I/R	K1_Ert	S/I/R	MGH	S/I/R	MGH_Mero	S/I/R
Amikacin	32	R	32	R	32	R	32	R	2	S	2	S
Ampicillin	>512	R	>512	R	>512	R	>512	R	>512	R	>512	R
Aztreonam	>256	R	64	R	>256	R	>256	R	ND	ND	ND	ND
Cefoxitin	64	R	>128	R	>128	R	>128	R	16	R	64	R
Ceftazidime	>128	R	>128	R	>128	R	>128	R	1	S	1	S
Chloramphenicol	64	R	64	R	64	R	64	R	512	R	512	R
Ciprofloxacin	>256	R	64	R	256	R	>256	R	2	R	2	R
Colistin	1	S	1	S	1	S	1	I	1	S	1	S
Ertapenem	1	S	64	R	64	R	64	R	0.25	S	1	I
Gentamicin	128	R	128	R	128	R	128	R	16	R	16	R
Imipenem	0.125	S	4	I	4	I	4	I	0.25	S	0.5	S
Meropenem	0.125	S	8	R	8	R	8	R	0.125	S	0.5	S
Piperacillin	>512	R	>512	R	>512	R	>512	R	128	R	128	R
Sulbactam	>32	R	>32	R	>32	R	>32	R	32	R	>32	R
Tetracycline	16	R	8	R	16	R	16	R	>256	R	>256	R
Tigecycline	4	R	2	I	2	I	2	I	0.25	S	0.25	S
Tobramycin	64	R	64	R	64	R	128	R	16	R	16	R

MIC values are in *mg*/L.
S, sensitive; I, intermediate; R, resistant. ND, not determined.

TABLE 3. Ertapenem disk susceptibility testing in the presence/absence of clavulanic acid (Clav). Diameters are in millimetres

	K1	K2	CTX-M-15 (1)	CTX-M-15 (2)	ATCC 13883	MGH78 578
Ertapenem	26	0	30	28	38	40
Ertapenem/Clav	31	12	37	36	38	40
Difference	5	12	7	8	0	0

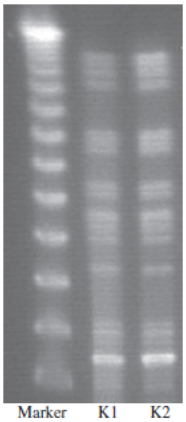

FIG. 1. The pulsed-field typing of K1 and K2 after digestion with *Xba*I.

β-Lactamase detection

Both strains were shown to contain the β-lactamases CTX-M-15, OXA-1, TEM-1 and SHV-1, but no carbapenemases were detected. The modified Hodge test showed no indication of carbapenemase activity.

OMP analysis and sequencing

OMP analysis showed that both K1 and K2 lacked a major OMP of around 40 kDa, assumed to be OmpK35, when compared with the profile of ATCC 13883. K2 was also shown to lack another protein of around 36 kDa, compatible with OmpK36 (Fig. 2). The sequencing of *ompK35* and *ompK36* from K1, K2 and ATCC 13883 revealed a number of mutations in both genes when compared with ATCC 13883 but identical sequences between the two strains. Therefore the 36 kDa protein found lacking in K2 cannot be

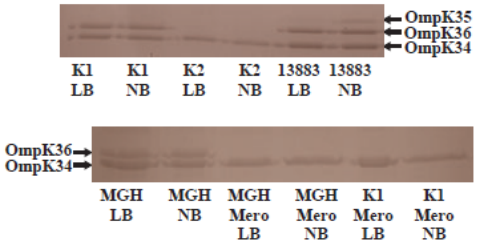

FIG. 2. A section of an SDS PAGE gel showing the major OMPs in K1, K2, ATCC 13883, MGH78578, an MGH78578 meropenem mutant and a K1 meropenem mutant. NB, nutrient broth; LB, Luria-Bertani broth.

explained by the mutations found in the *ompK36* gene sequence.

Mutation studies

Both K1 ertapenem and meropenem mutants were shown to exhibit a similar resistance and identical OMP phenotype to that of K2 (Table 2 and Fig. 2). MGH78578 meropenem mutants exhibited porin loss similar to that of the K1 mutants and K2 strain, and showed an increase in MICs of ertapenem from 0.25 to 1 mg/L. An increase from 0.25 to 0.5 mg/L was observed in the MIC of imipenem and an increase from 0.125 to 0.5 mg/L was observed in the MIC of meropenem. It is unlikely that porin loss alone can be responsible for the carbapenem resistance exhibited by K2.

RT-PCR

RT-PCR analysis of K1, K2 and ATCC 13883 showed that *ramA* and *acrA* were upregulated in both K1 and K2. This suggests that much of the MDR phenotype exhibited by both K1 and K2 may be attributed to active efflux mechanisms. Analysis of the OMP gene expression showed that *ompK35* was not expressed in either strain and *ompK36* was expressed at similar levels in each strain (Fig. 3). This suggests that the OMP absent in both K1 and K2 was indeed OmpK35 and that the absence of OmpK36 in K2 may be due to a post-transcriptional process. It is likely that *ompK35* expression is inhibited as a consequence of *ramA* overexpression, as has been found previously [26].

ramR sequencing

The sequencing of *ramR*, the repressor gene of *ramA*, revealed that both K1 and K2 contained identical mutations, which resulted in a premature stop codon at amino acid position 83. RamA is a known regulator of the AcrAB efflux pump; therefore the *ramA* and subsequent *acrA* overexpression are likely to be attributable to this mutation [27].

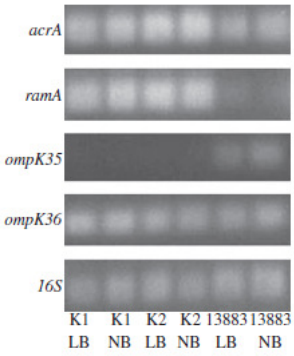

FIG. 3. The expression of the selected genes in K1, K2 and ATCC 13883. NB, nutrient broth; LB, Luria-Bertani broth.

Transconjugation studies and plasmid profiling

K1/J62-2 transconjugants were obtained by selection with rifampicin and gentamicin, but no transconjugants were obtained with ceftazidime or meropenem. Susceptibility testing showed that the transconjugants were resistant to gentamicin and ceftazidime but not to carbapenems. PCR analysis of the transconjugant strains showed that the transferred plasmid contained CTX-M-15, TEM-1 and OXA-1 β-lactamases. No K2/J62-2 transconjugants could be obtained. S1 nuclease plasmid profiling showed that K1 and K2 both contained plasmids of *c.* 194, 97 and 70 kb, and K1 also appeared to have an additional plasmid of <48 kb that was not present in K2. Analysis of the transconjugant strains showed that the plasmid of *c.* 97 kb had been transformed into the recipient strain, indicating that CTX-M-15, TEM-1 and OXA-1 β-lactamase genes are present on the same plasmid. The ability to select for the transconjugants with gentamicin indicates that the plasmid additionally carries an aminoglycoside resistance determinant.

Discussion

Although the production of dedicated carbapenemases is often viewed as the primary cause of carbapenem-resistant *K. pneumoniae* infections, this study has shown that carbapenem resistance resulting in failed therapy is possible in the absence of such enzymes. The importance of permeability changes and the presence of β-lactamases, not normally associated with carbapenem resistance, may have been underestimated regarding their importance in conferring levels of resistance that can be detrimental to infection treatment. It has previously been suggested that CTX-M-15 may be capable of contributing towards carbapenem resistance through the hydrolysis of ertapenem, which this study supports [5]. The reduced ertapenem susceptibility levels observed in strain K1 could be attributed to the presence of the plasmid-borne β-lactamase, CTX-M-15. Although the strain in question additionally contained other β-lactamase genes (OXA-1, TEM-1, SHV-1), only the CTX-M-15 β-lactamase has previously been reported to exhibit a degree of carbapenemase activity and, in this case, it seems likely that CTX-M-15 β-lactamase plays a contributory role in exerting the carbapenem-resistant phenotype, specifically to ertapenem.

The contribution of efflux mechanisms to carbapenem resistance is currently a disputed topic, although efflux undoubtedly plays an important role in conferring the MDR phenotype displayed by the isolates in this study [11,28]. Although the precise effect of active efflux in exporting carbapenems from bacterial cells remains unknown, the upregulation of the transcriptional activator *ramA* and subsequently the RND family efflux pump gene, *acrA*, present in both strains, may contribute to the high ertapenem MICs exhibited. Resistance to ertapenem has been associated with a combination of efflux mechanisms and permeability changes in a study of clinical strains of *E. cloacae* [9]. It is possible that ertapenem may be a suitable substrate for this efflux system.

Expression of the major OMPs is certainly an important factor for the access of various classes of antibiotics into the bacterial cell and it appears that in *K. pneumoniae*, OmpK36 is of particular importance for carbapenem permeability. The absence of this porin alone was shown to confer intermediate resistance to ertapenem and reduce susceptibility to meropenem in the control strain MGH78578, and the absence of this porin appears to be the sole factor in the conversion of K1 into K2. These results suggest that OmpK36 acts as the primary channel for the passage of ertapenem and meropenem but not for imipenem. It has been shown that imipenem is capable of penetrating the bacterial cell wall faster than meropenem [29], probably due to a combination of its zwitterionic charge and smaller size, and this may allow imipenem to penetrate other porins that meropenem and ertapenem cannot.

No carbapenemases were found in either *K. pneumoniae* strain despite being subjected to PCR detection using primers for all known carbapenemases found in Gram-negative bacteria. This finding alongside the maintained imipenem susceptibility in K2 suggests that a carbapenemase is not responsible for conferring the observed phenotype but rather a combination of the aforementioned resistance mechanisms. Perhaps the most important feature to note is the ease by which porin-deficient mutants could be selected, particularly with ertapenem, which could inadvertently make any subsequent therapy with meropenem less effective or even defunct. Such findings are particularly worrying when considering that, in the incidence of the spread of a similar *K. pneumoniae* clone, such infections would be untreatable in UK hospitals, where the only effective drugs, colistin and imipenem, are not typically used.

This study demonstrates the interplay between different resistance mechanisms that are capable of achieving levels of carbapenem resistance, in the absence of a carbapenemase, that are sufficient to lead to therapy failure.

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Transparency Declaration

The authors have no conflict of interest to declare.

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JMM Editorial

Carbapenems: do they have a future?

The pivotal role of glycopeptides in the control of meticillin-resistant *Staphylococcus aureus* is well known and the notoriety of this organism has ensured that new antibacterial treatments have been developed. Far less publicized is the crisis in the treatment of Gram-negative infections, especially those that are hospital-acquired. In many cases, particularly because of the emergence and spread of extended-spectrum β-lactamases (ESBLs) in the *Enterobacteriaceae* and the increased isolation of non-fermenting bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, the remaining standard treatment option has been a group of penem antibiotics with a carbon atom in the side ring rather than the traditional sulphur. These are the carbapenems.

In the UK, there are three main carbapenems, imipenem, meropenem and ertapenem, and a new addition, doripenem. Imipenem is a broad-spectrum antibiotic, derived from a compound isolated from the soil bacterium *Streptomyces cattleya* (Kahan *et al.*, 1983), which has to be co-administered with cilastatin to prevent its degradation by the kidney. Meropenem is also a broad-spectrum β-lactam, and has a therapeutic advantage over imipenem because it can be used to treat central nervous system infections. The addition of a methyl group in the 1-position of the carbapenem moiety in meropenem makes it structurally different from imipenem (Wiseman *et al.*, 1995) and this modification enhances the *in vivo* stability of meropenem, compared to imipenem, and it does not need to be co-administered with cilastin (Edwards, 1995). Both compounds are used as empiric therapy for a wide range of severe Gram-negative infections. Ertapenem has a more limited spectrum and has largely been recommended for the treatment of community-acquired infections, especially those caused by bacteria carrying an ESBL. It can partially inhibit non-fermenting bacteria and thus would select those strains that have a pre-disposition to carbapenem resistance (Amyes *et al.*, 2007). This reason questions its use in the treatment of hospital-acquired infections as it may preferentially select non-fermenting bacteria. Doripenem has a profile similar to that of imipenem and meropenem and does not overcome the majority of currently prevalent mechanisms of resistance to the carbapenems.

Mechanism of resistance

Unfortunately, resistance has emerged in many bacteria treated with carbapenems. The most common mechanisms of resistance are the acquisition of carbapenem-hydrolysing β-lactamases of Ambler class D enzymes (oxacillinases) (CDO) (Poirel & Nordmann, 2002), β-lactamases belonging to class B (metallo-enzymes) (MBLs) (Walsh *et al.*, 2005) and a few class A β-lactamases such as the KPC enzymes in *Klebsiella* species. Often these β-lactamases do not act alone and are often accompanied by mutations in genes encoding penicillin-binding proteins and alteration in outer-membrane permeability; for example, the loss of porins CarO and Omp33–36 in *A. baumannii* (Gehrlein *et al.*, 1991).

In *A. baumannii*, the spread of carbapenem resistance largely results from the clonal dissemination of a resistant strain where a crucial combination of a mobile carbapenem resistance gene (often encoding the class D β-lactamases OXA-23 or OXA-58) has entered a congenial host (Brown & Amyes, 2006). The spread of these resistant bacteria is due as much to cross-infection as to antibiotic usage. These genes have migrated to the congenial host because they are closely linked to insertion sequences, which have promoted their mobility (Turton *et al.*, 2006; Poirel & Nordmann, 2006). A further complication is that all *A. baumannii* possess an inherent class D β-lactamase, collectively known as OXA-51-like, which can provide weak hydrolytic activity on the carbapenems, though currently only rarely produces clinical resistance.

There are five groups of acquired MBLs (IMP-like, VIM-like, SIM-1, SPM-1 and GIM-1 enzymes). These have largely been found in non-fermenting bacteria; for instance the first three have been identified in *A. baumannii* (Peleg *et al.*, 2008). They are less common in other Gram-negative bacteria. Recent concern has focussed on the emergence of resistance in the *Enterobacteriaceae*, particularly with the emergence of the NDM-1 β-lactamase in *Klebsiella* species. Coupled with the emergence of the KPC class A serine β-lactamases, this augurs badly for the carbapenems (Kumarasamy *et al.*, 2010). Unlike *A. baumannii*, the development and spread of resistance in *Klebsiella* species is less well defined and currently much rarer; the emergence of the mobile β-lactamase genes is still in its infancy and the crucial combination of this gene in a suitable host does not yet appear to have occurred. Thus the mobile genes are still migrating and this would be aided by imprudent therapy.

The importation of carbapenem-hydrolysing β-lactamases is not the only threat in *Klebsiella* species. ESBL-producing *Klebsiella pneumoniae* strains are now very common and the carbapenems are often the preferred course for treatment; in particular, ertapenem, a once daily parenteral 1-β-methyl carbapenem antibiotic, licensed in 2002 for the treatment of intra-abdominal and gynaecological infections and community-acquired pneumonia (Livermore *et al.*, 2003). It is considered a first-line antibiotic for complicated community-acquired infections and, as such, is often prescribed for the treatment of ESBL-producing coliform infections (Livermore *et al.*, 2003). It has been shown that the use of ertapenem in *K. pneumoniae* can select for the loss of the major outer-membrane protein OmpK36, resulting in reduced accumulation of ertapenem in the bacterial cell and subsequently reduced susceptibility (Girlich *et al.*, 2009). Studies in *K. pneumoniae* have shown that the loss of OmpK36 and the presence of non-carbapenemase β-lactamases, such as

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the ESBL CTX-M-15, are sufficient to exert resistance to ertapenem whilst causing a concomitant reduction in susceptibility to meropenem and imipenem (Doumith *et al.*, 2009; Jacoby *et al.*, 2004). The potential for cross-resistance is particularly worrying because if ertapenem is administered to a patient prior to other carbapenem treatment for a recurring infection, porin-deficient mutants may be inadvertently selected for, resulting in reduced susceptibility to the other carbapenems, which potentially can result in therapy failure.

Carbapenems, especially the broad-spectrum variants, are an extremely important part of our ability to control severe Gram-negative infections, particularly those caused by multidrug-resistant bacteria. However, resistance is emerging in the form of new β-lactamases able to migrate to clinically important strains and confer high levels of clinical resistance, a situation similar to that seen with the ESBLs two decades ago. The situation is more complicated; some species of bacteria (*A. baumannii*, *K. pneumoniae*, etc.) already possess β-lactamases that can, under certain conditions particularly with the assistance of reduced permeability, reduce the susceptibility of the bacterium to all carbapenems. The alternative to carbapenem therapy is the re-emergence of the polymyxins, such as colistin methanesulphate, which are considered a last resort salvage therapy (Li *et al.*, 2006). The ESBLs caused the decline of the cephalosporins; there are now sufficient β-lactamases to do the same to the carbapenems. Prudent therapy with carbapenems should prolong their efficacy and this should not be compromised by empiric therapy with less-active drugs. The alternative would be an increased reliance on colistin and that would appear a poor substitute if we allow the carbapenems to join the large group of excellent antibiotics that we once had to treat severe Gram-negative infections.

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Epidemiology of *Acinetobacter baumannii* of animal origin☆

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ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen responsible for nosocomial infections, however the origins of these bacteria remain unclear. Sixteen *A. baumannii* strains collected from animals slaughtered for human consumption were investigated for their susceptibility profiles, resistance islands (RIs), class 1 integrons, insertion sequence IS*Aba1*, and bla_{OXA-51}-like and bla_{AmpC} genes. Polymerase chain reaction (PCR) and sequencing approaches were used to identify and type the isolates using the intrinsic gene bla_{OXA-51}-like genes. Genotyping was also performed by pulsed-field gel electrophoresis (PFGE) to establish whether there was a genetic relationship between animal isolates and the main human isolates of European clones I, II and III (ECI, ECII and ECIII) known to cause major hospital outbreaks. All 16 isolates (100%) were sensitive to carbapenems, gentamicin, ciprofloxacin and piperacillin/tazobactam but were resistant to amoxicillin, cefradine, trimethoprim and chloramphenicol. Moreover, all isolates had a baseline resistance to ceftazidime, with a minimum inhibitory concentration of 4 mg/L. All isolates lacked RIs, IS*Aba1* and class 1 integrons but harboured bla_{OXA-51}-like and bla_{AmpC} genes. In addition, this study reports for the first time three new bla_{OXA-51}-like genes ($bla_{OXA-148}$, $bla_{OXA-149}$ and $bla_{OXA-150}$) isolated from bacteria in cattle, which have not been found previously in human isolates. However, all isolates recovered from pig faecal samples harboured one type of bla_{OXA-51}-like (bla_{OXA-51} itself), which has already been reported in human clinical isolates. From sequencing of the bla_{OXA-51}-like genes from animal isolates, it was possible to identify four different clusters similar to those identified by PFGE, which in turn also distinguished these four groups from the human ECI, ECII and ECIII strains.

1. Introduction

Acinetobacter baumannii is an opportunistic pathogen responsible for a wide range of nosocomial infections including meningitis, bacteraemia and pneumonia, especially in the Intensive Care Unit (ICU) [1]. It is responsible for 2–10% of all Gram-negative bacterial infections in ICUs in Europe [2] and can be difficult to treat owing to the increasing incidence of resistance to carbapenems, which in many cases represent the final treatment option [3].

Fournier et al. [4] have recently identified the largest resistance island (RI) ever found (86 kb), in a strain of *A. baumannii* (AYE), containing a cluster of 45 genes of which 25 are associated with various classes of antibiotics such as β-lactamases, aminoglycosides, tetracycline, trimethoprim and sulfamethoxazole, and chloramphenicol. In addition, the sequence of this RI showed that it was constructed by broad host-range mobile genetic elements (integrons, transposons and gene cassettes from class 1 integrons) [4]. Of interest, class 1 integrons, which can be part of a RI as mentioned previously or located on the chromosome and presumed to be transported there [5], are prevalent amongst *Acinetobacter* spp. and have been described as vehicles for the acquisition of resistance genes, which could then spread to other pathogens in the hospital settings [6,7]. A more recent publication by Shaikh et al. [8] has shown that many *A. baumannii* clinical isolates have RIs inserted in their genomes.

Previous studies have indicated that the presence of an insertion sequence upstream of the $bla_{OXA-51-like}$ and bla_{AmpC} genes is the factor responsible for the expression of resistance to carbapenems [9,10] and ceftazidime [11–13], respectively.

Antibiotics have been widely used as growth promoters in livestock production or to control infectious disease, and this treatment has been linked to the emergence and dissemination of resistant bacteria, which can then be passed to people via food or by direct contact with infected animals [14]. Although a plethora of work has been done over the last two decades to understand the origin of hospital-acquired infections, information on the sources of *A. baumannii* is still lacking.

The aim of this study was to determine whether *A. baumannii* strains collected from animals slaughtered for human consumption from Scottish abattoirs possessed major epidemiological characteristics similar to strains isolated from clinical patients.

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Table 1
Antimicrobial susceptibility of 16 animal isolates of *Acinetobacter baumannii* to various antimicrobial agents.

Isolate ID	MIC (mg/L)										
	AMX	CTZ	CEF	IPM	MER	TZP	GEN	CIP	TMP	CHL	TET
PF07	>128	4	128	0.12	0.25	16	0.25	0.12	4	>128	4
PF16	>128	4	128	0.12	0.25	16	0.25	0.12	4	>128	4
PF18	>128	4	128	0.12	0.25	16	0.25	0.12	8	>128	4
PF19	>128	4	128	0.12	0.25	16	0.25	0.12	8	>128	4
PF20	>128	4	>128	0.12	0.25	16	0.25	0.12	4	>128	4
PF33	>128	4	>128	0.5	0.25	16	4	0.12	8	>128	4
PF34	>128	4	>128	0.12	0.25	16	0.25	0.12	4	128	4
PF36	>128	4	>128	0.12	0.25	16	0.25	0.12	8	128	4
CF233	>128	4	>128	0.12	0.25	16	0.25	0.25	16	128	4
CF234	>128	4	>128	0.12	0.25	16	0.25	0.25	16	128	4
CF251	>128	4	>128	0.12	0.25	16	0.5	0.25	8	128	4
CF254	>128	4	>128	0.12	0.25	16	0.5	0.25	16	128	4
CF258	>128	4	>128	0.12	0.25	16	0.5	0.25	16	128	2
CF260	>128	4	>128	0.12	0.25	16	0.5	0.25	16	128	8
CN26	>128	4	>128	0.5	0.25	16	04	0.25	8	128	4
CN35	>128	4	>128	0.12	0.25	16	0.5	0.25	8	128	4

MIC, minimum inhibitory concentration; AMX, amoxicillin; CTZ, ceftazidime; CEF, cefradine; IPM, imipenem; MER, meropenem; TZP, piperacillin/tazobactam; GEN, gentamicin; CIP, ciprofloxacin; TMP, trimethoprim; CHL, chloramphenicol; TET, tetracycline.

2. Materials and methods

2.1. Bacterial strains, sampling and species identification

Sixteen *A. baumannii* isolates (eight from pigs and eight from cattle) were obtained from an original group of 1381 animal samples collected between the period February 2006 to August 2007, from recently slaughtered animals destined for human consumption and collected at major Scottish abattoirs [15]. Isolates were recovered from faecal specimens, skin, nostril and ear swabs. Furthermore, some of the faecal samples were taken from food-producing cattle from 31 farms, randomly selected from a list of 3111 farms provided from the 1997 Scottish Agricultural and Horticultural Census data, stratified by geographical region [15]. Each sample presented here came from an individual animal from a discrete farm in Scotland.

Acinetobacter baumannii control isolates used in this study were as follows: AYE strain, a positive control for RI, bla_{AmpC} genes, bla_{OXA-51}-like and insertion sequence IS*Aba1*; and European clones I, II and III (ECI, ECII and ECIII) for pulsed-field gel electrophoresis (PFGE) comparison. Species identification was carried out by polymerase chain reaction (PCR) analysis of the 16S–23S rRNA intergenic spacer sequences [16] and the presence of a bla_{OXA-51}-like gene [17].

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of amoxicillin, ceftazidime, cefradine imipenem, meropenem, piperacillin/tazobactam (TZP), gentamicin, ciprofloxacin, trimethoprim, chloramphenicol and tetracycline were determined by the agar dilution method following British Society for Antimicrobial Chemotherapy (BSAC) guidelines [18]. The accuracy of the technique was checked using the control strains *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* NCTC 6571. MIC analysis was performed according to BSAC breakpoints [18], except for tetracycline where the breakpoint has not been determined by BSAC and therefore was according to the Clinical and Laboratory Standards Institute (CLSI) (MIC ≥ 16 mg/L) [19] (Table 1).

2.3. Polymerase chain reaction and sequencing

PCR was used to amplify the following genes with their specific primers as follows: bla_{OXA-51}-like, OXA-69A (5′-CTAATAATTGATCTACTCAAG-3′), OXA-69B (5′-CCAGTGGATGGATGGATAGATTATC-3′) [20]; bla_{AmpC}, ADC-1 (5′-CCGCGACAGCAGGTGGATA-3′), ADC-2 (5′-TCGGCTGATTTTCTTGGTT-3′) [21]; IS*Aba1*, ISAba1, 5′-CATTGGCATTAAACTGAGGAGAAA-3′, ISAba2, 5′-TTGGAAATGGGGAAAACGAA-3′ [17]; and class 1 integrons, intI1L (5′-ACATGTGATGGCGACGCACGA-3′), intI1R (5′-ATTTCTGTCCTGGCTGGCGA-3′) [6]. PCR products were analysed by agarose gel (1.5%, w/v) electrophoresis followed by staining with GelRed™ (Biotium, Cambridge Bioscience, Cambridge, UK) and visualisation using the Gel Doc™ software image capturing system (Bio-Rad, Hemel Hempstead, UK). Following purification using a QIAquick® PCR purification kit (QIAGEN, Crawley, UK), bla_{OXA-51}-like products were sequenced in both directions on a 3730 DNA analyzer (Applied Biosystems, Warrington, UK). The resulting sequences were analysed using the online software BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/).

Identification of RI-positive isolates was achieved by PCR as described previously with primers 2F (5′-TCCATTTTACCGCCACTTTC-3′) and 4R (5′-AATCGATGCGGTCGAGTAAC-3′) [8]. Briefly, amplification of a 450-bp product indicates the absence of a RI, however the lack of a PCR amplicon across the insertion site of the ATPase gene may indicate the presence of a RI [8].

2.4. Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA was prepared and digested with *Apa*I (Promega, Southampton, UK) as described previously with modification [22]. DNA fragments were separated on 1%, w/v agarose gels in 0.5× TBE buffer [1× TBE buffer comprises 89 mM Tris, 89 mM boric acid and 2 mM ethylene diamine tetra-acetic acid (EDTA)] at 14 °C using a CHEF DRII apparatus (Bio-Rad) with 6 V/cm, pulsed from 5 s to 35 s for 24 h. Gels were stained with ethidium bromide and were scanned using the Bio-Rad Gel Doc software image capturing system. The Dice coefficient was used to calculate similarities, and the unweighted pair group method using average linkages (UPGMA) was used for cluster analysis with BioNumerics software v. 4.0 (Applied Maths, St Martens-Latem, Belgium). Isolates that clustered together with a similarity of >82% were considered to belong to the same PFGE type.

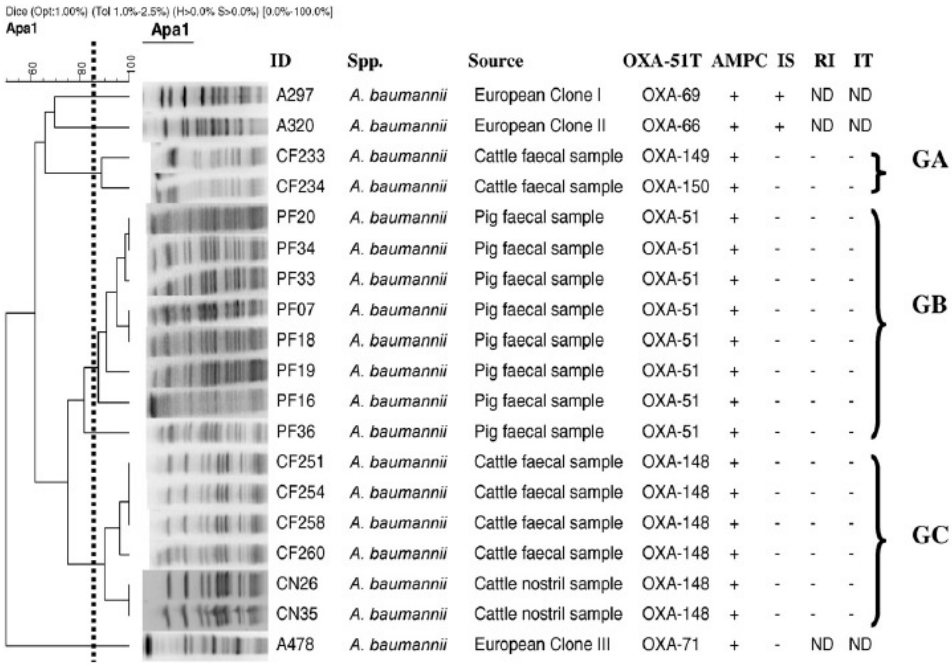

Fig. 1. Pulsed-field gel electrophoresis (PFGE) dendrogram to illustrate the relatedness of *Acinetobacter baumannii* isolates collected from animals and major European humans clones ECI, ECII and ECIII [23]. The percentage similarity scale is shown above the dendrogram. The dotted vertical line indicates the >82% similarity threshold. The Dice coefficient was used to calculate similarities, and the unweighted pair group method using average linkages (UPGMA) was used for cluster analysis with BioNumerics software v. 4.0. OXA-51T, bla_{OXA-51} type; IS, IS*Aba1*; RI, resistance island; IT, class 1 integrons; ND, not determined; +, positive; –, negative.

3. Results

3.1. Species identification and antimicrobial susceptibility testing

All pig ($n = 8$) and cattle ($n = 8$) isolates harboured bla_{OXA-51}-like genes, indicating that they were *A. baumannii*. PCR analysis of the 16S–23S rRNA intergenic spacer sequences confirmed these results (Fig. 1) [16]. All 16 strains (100%) were previously shown to be sensitive to imipenem (MIC ≤ 4 mg/L), meropenem (MIC ≤ 4 mg/L), ciprofloxacin (MIC ≤ 0.5 mg/L) and TZP (MIC ≤ 16 mg/L) but had a baseline resistance to ceftazidime (MIC > 2 mg/L) [12] (Table 1). In this work, these strains were further investigated for their susceptibility to a wide range of antibiotics and all (100%) were resistant to amoxicillin (MIC > 16 mg/L), cefradine (MIC > 8 mg/L) and chloramphenicol (MIC > 8 mg/L) but susceptible to tetracycline (MIC < 16 mg/L) and gentamicin (MIC < 4 mg/L).

3.2. Polymerase chain reaction

In addition to harbouring the bla_{OXA-51}-like gene, all 16 *A. baumannii* isolates harboured bla_{AmpC} genes; however, all lacked IS*Aba1* upstream of either gene as well as class 1 integrons (Fig. 1). The presence of PCR amplification products of 450 bp with the 2F and 4R primers demonstrated the absence of RIs.

3.3. Analysis of bla_{OXA-51}-like sequencing

Sequencing of bla_{OXA-51}-like revealed that all *A. baumannii* isolated from pig faecal samples ($n = 8$) harboured only bla_{OXA-51} type; however, the 8 isolates collected from cattle had three new gene variants named $bla_{OXA-148}$ ($n = 6$; 4 from faecal samples and 2 from nostril samples), $bla_{OXA-149}$ (from a faecal sample) and $bla_{OXA-150}$ (from a faecal sample) (Fig. 1). Comparison of OXA-51 types found in animal isolates revealed that OXA-149 and OXA-150 were closely related, with only one amino acid change ($Q_{194}P$) (Fig. 2) However, OXA-148 differed by five, six and seven amino acids from OXA-150, OXA-149 and OXA-51, respectively (Fig. 2). When positioned in the map of Evans et al. [23] (Fig. 3), isolates with the closely related enzymes OXA-149 and OXA-150 clustered together, however those with OXA-148 and OXA-51 as well as OXA-69, OXA-66 and OXA-71 from ECI, ECII and ECIII formed their own individual group (Fig. 3).

3.4. PFGE analysis and its correlation with the sequence of bla_{OXA-51}-like gene

Compared with *A. baumannii* clinical isolates ECI, ECII and ECIII, the pig and cattle isolates had different PFGE patterns and were grouped in three different clusters (A, B and C) with similarity percentage ranging between 82% and 89.93% (Fig. 1). Each individual cluster had its specific OXA-51-like enzyme: cluster A had OXA-149 and its related enzyme OXA-150; and clusters B and C had OXA-51 and OXA-148, respectively (Fig. 1). The ECI, ECII and ECIII with their corresponding OXA-69, OXA-66 and OXA-71 enzymes grouped separately from these three clusters.

4. Discussion

Acinetobacter baumannii is one of the most important pathogens causing hospital-acquired infections worldwide [24]. To our knowledge, this is the first study investigating the epidemiology of *A.*

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OXA-148* MNIKALLLIT SAIFISACSP...T..........KKAEKIKNLFNEAHTTGVLVIQQGQTQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKASTTEVFKWNGQKRL
OXA-149* .......... ..........S..........E.....................................................T.............
OXA-150* .......... ..........S..........E.....................................................T.............
OXA-66   .......... ..........V..........E.....................................................T.........K...
OXA-69   .......... ..........D..........E.....H...............................................T.........E...
OXA-51   ....T..... ..........E..........V.....E.....................................................T.............
OXA-71   .......... ..........E..........E.....................................................TT............

OXA-148  FPEWEKDMTLGDAMKASAIPVYQDLARRIGLELMSNEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQFAYKLANKTLPFSQKVQDEVKSMLFIEEKNGNKIYAKSGW
OXA-149  ..............................H.....................................................P......Q...................
OXA-150  ..............................H.....................................................P......Q...................
OXA-66   ..............................K.....................................................P......Q...................
OXA-69   ..............................K.....................................................P......Q...................
OXA-51   ..............................K.....................................................P......Q...................
OXA-71   ..............................K.....................................................P......Q...................
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Fig. 2. Comparison of the amino acid sequences of the partial $bla_{OXA-148}$, $bla_{OXA-149}$, $bla_{OXA-150}$ and bla_{OXA-51} genes isolated from animal isolates with bla_{OXA-69}, bla_{OXA-66} and bla_{OXA-71} from European clones ECI, ECII and ECIII. Protein sequences from the European clones were obtained based on the indicated GenBank accession nos.: bla_{OXA-51} (Q5QT35); bla_{OXA-66} (AAW81338.1); bla_{OXA-69} (AAW81340.1); and bla_{OXA-71} (AAW81342.1). * Indicates new genes.

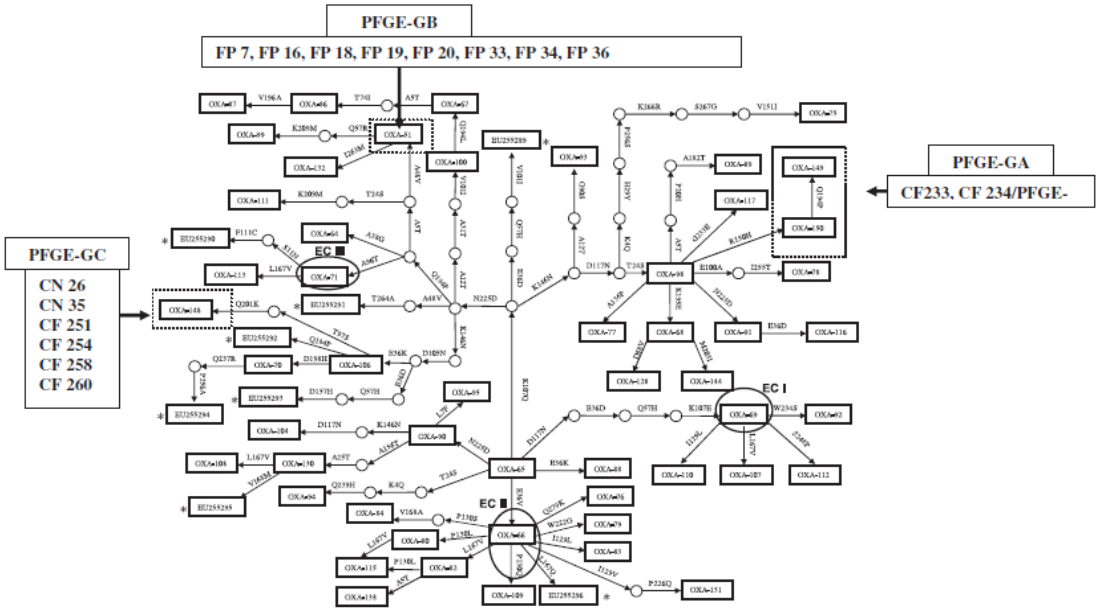

Fig. 3. Modified OXA-51-like enzyme map by Evans et al. [23]. Enzymes found in European clones ECI, ECII and ECIII are circled with a plain line. $bla_{OXA-148}$, $bla_{OXA-149}$, $bla_{OXA-150}$ and bla_{OXA-51} are highlighted in dashed boxes. Pulsed-field gel electrophoresis (PFGE) groups A, B and C are highlighted in plain boxes. * The PubMed accession no. is listed for these enzymes as their official designation has yet to be publicly released [23].

baumannii recovered from animal samples to establish whether there is a link between these isolates and those associated with infectious disease in humans. It could be considered surprising that so many of the strains were similar to one another, bearing in mind that they came from different farms. However, there is considerable movement of animals between farms, in particular many animals are sourced from relatively few suppliers.

Results of susceptibility testing showed that all 16 isolates were susceptible to first-line antibiotics used to treat infections caused by *A. baumannii*, such as imipenem and meropenem, although there are many mechanisms of resistance to carbapenems in this organism; the susceptibilities observed can be explained by the lack of IS*Ab1* upstream of the relevant genes. Moreover, ciprofloxacin, TZP, gentamicin and tetracycline were active against these strains and this differs from the susceptibility patterns found in contemporary clinical isolates where resistance to these compounds is now widespread [25].

It is also noteworthy to highlight the fact that *Acinetobacter* strains are naturally resistant to cephalosporins owing to production of a cephalosporinase (AmpC) that is known to hydrolyse amino-penicillins and first-, second- and third-generation cephalosporins and has a low catalytic activity against ceftazidime [26]. Therefore, the resistance observed for amoxicillin, cefradine and ceftazidime (low-level resistance, MIC > 2 mg/L) can be attributed to this gene. Resistance to trimethoprim (MIC > 2 mg/L) was expected as *A. baumannii* is inherently resistant to this compound because the target protein, dihydrofolate reductase, has a low affinity for the drug [27]. In contrast, chloramphenicol resistance (MIC > 8 mg/L) was not expected and this antimicrobial may have been used in these animals either as a growth promoter, prophylactically or for treatment.

The recent discovery and spread of large RIs in many *A. baumannii* clinical isolates has altered our concept of how these strains have become multidrug-resistant (MDR) [4,8]. Moreover, class 1

integrons play an important role in the acquisition and spread of antibiotic resistance in *A. baumannii* clinical isolates. Therefore, in this study the *A. baumannii* animal isolates were screened for the presence of these mobile genetic elements and, as expected, they were free from RIs and class 1 integrons. These results correlate well with the absence of RIs, where these integrons often reside. The absence of these resistance-driven mobile elements in the animal isolates suggests that no or little antibiotic selective pressure was applied to these pathogens, contrary to their counterpart from the clinical setting.

Many techniques have been developed for *A. baumannii* identification but they are costly, laborious and time consuming. bla_{OXA-51}-like genes are naturally occurring in *A. baumannii* [20] and are used as markers in a simple PCR-based assay for identification of this species isolated from clinical samples [17]. One of the aims of this work was to test whether the same assay can be used to recognise previously identified *A. baumannii* of animal origin [15]. Although the proportion of these isolates is relatively low in the animal population (1.2%), all isolates were positive for bla_{OXA-51}-like genes and therefore this technique could also be used, at least as a presumptive method for identification of *A. baumannii* isolated from animal samples.

We have previously shown that analysis of bla_{OXA-51} gene sequencing could be used to identify clonal lineages of outbreak strains ECI, ECII and ECIII [23]. Analysis of OXA-51-like enzymes revealed that the representative enzymes found in animal isolates defined four different groups (OXA-149 and OXA-150, OXA-51, and OXA-148). This may be explained by the fact that these groups differed between themselves by five to seven amino acids, with OXA-149 and OXA-150 being the most closely related enzymes and therefore grouped together. In addition, PFGE clearly divided the animal strains and ECI, ECII and ECIII into four clusters that agreed with the OXA-51 types typing method (Fig. 1).

In conclusion, these data provide additional evidence that MDR *A. baumannii* found in hospitals may not have evolved from their animal counterpart. The animal isolates were not MDR and lacked significant antibiotic resistance features such as RIs, class 1 integrons and IS*Aba1*. Moreover, we have shown that many of the bla_{OXA-51}-like genes are also intrinsic in animal isolates and can be used not only for species identification but also for presumptive typing. These results, coupled with the different PFGE profiles of *A. baumannii* animal isolates from human strains, suggest that they are not the precursors of *A. baumannii* strains found to cause hospital-acquired infections.

Amino acid sequence accession nos.

The sequence data reported in this paper have been deposited in GenBank under the accession nos. ACX31140.1, ACX31141.1 and ACX31142.1.

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Competing interests: None declared.

Ethical approval: Not required.

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